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Investigation of Effects of Prolonged Inhalation of Nickel-Enriched Fly Ash in Syrian Golden Hamsters

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Groups of 102 Male Syrian Golden hamsters were chronically exposed to ~70 µg/ℓ respirable Nickel Enriched Fly Ash aerosol (high NEFA group), ~17µ g/ℓ (low NEFA group), or ~70 µgℓ FA 6 hrs/day, 5 days/week for 4, 8, 12, 16 and 20 months. Identical control groups received sham exposures. The NEFA particles of respirable size contained approximately 6% nickel, compared to about 0.3% for FA.

Exposure to NEFA had no significant effect on the apparent well being, body weight and life span of the animals although heavy deposits of NEFA in the lungs were found. The lung weights and mean lung volumes of the high NEFA and FA exposed animals were significantly higher and larger, respectively, than for the low NEFA and control groups. There was a 100% incidence of dust deposition (anthracosis) and significantly higher incidence plus severity of interstitial reaction and bronchiolization in the dust-exposed groups than the controls. The severity of lung reactions was significantly lower in the low NEFA group than the high NEFA and FA groups.

The results of this study conclude that the addition of nickel to fly ash under these conditions did not significantly ($P< 0.05$) enhance the pathogenicity (including carcinogenicity) of fly ash in this animal model.

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INVESTIGATION OF EFFECTS OF PROLONGED INHALATION OF NICKEL-ENRICHED FLY ASH IN SYRIAN GOLDEN HAMSTERS

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FOREWORD

The many benefits of our modern developing, industrial society are accompanied by certain hazards. Careful assessment of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our nations population.

The Health Effects Research Laboratory, Cincinnati, was established to provide sound health effects data in support of the regulatory activities of the EPA. An important segment of this data bank is the knowledge of the health effects of long-term inhalation of combustion products from electric power generating plants.

This report presents the results of a study to evaluate the potential toxicologic and carcinogenic effects of exposure to ordinary fly ash compared with fly ash enriched with nickel. By enriching the fly ash with nickel, the contribution of this element to the overall toxicity and/or carcinogenicity of fly ash can be assessed.

R.J. Garner
Director
Health Effects Research Laboratory

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ABSTRACT

Groups of 102 Male Syrian Golden hamsters were chronically exposed to $\sim 70 \mu g/\ell$ respirable Nickel Enriched Fly Ash aerosol (high NEFA group), $\sim 17 \mu g/\ell$ (low NEFA group), or $\sim 70 \mu g/\ell$ FA 6 hrs/day, 5 days/week for up to 20 months. An identical control group received sham exposures. The NEFA particles of respirable size contained approximately 6% nickel, compared to about 0.3% for FA. Five hamsters/group were sacrificed after 4, 8, 12 or 16 months of exposure. An additional 5 hamsters/group were withdrawn from exposure at the same intervals for lifelong observations.

Exposures to NEFA had no significant effect on the apparent well-being, body weight and life span of the animals although heavy deposits of NEFA in the lungs were demonstrated. The lung weights of the high NEFA- and of the FA-exposed animals, however, were significantly ($P < 0.01$) higher than those of the low NEFA controls. There was no significant difference between the mean body weights of the high NEFA group and the FA group. The mean lung volumes were significantly ($P < 0.01$) larger for the high NEFA group and the FA group than for the low NEFA group and the controls.

There was a 100% incidence of dust deposition, referred to in this report as anthracosis, in the lungs of exposed hamsters. Incidence and severity of interstitial reaction and bronchiolization were significantly higher in the dust-exposed groups than in the sham-exposed controls. The severity of anthracosis, interstitial reaction and bronchiolization was significantly lower ($P < 0.01$) in the low NEFA group than in the high NEFA and FA groups. This dose-effect relationship reflects the two different dose groups, namely low NEFA versus high NEFA and FA. While two malignant primary thorax tumors were found in two hamsters of the high NEFA group, no statistically significant carcinogenesis was observed. Of the exposure-related changes, only anthracosis decreased as a function of recovery time.

Comparison of pulmonary nickel burdens after 20 months of exposure with the aerosol concentration suggests that the pulmonary clearance rate was slower in the high NEFA exposure group than in the low NEFA exposure group.

The results of this study lead to the conclusion that the addition of nickel to fly ash under these experimental conditions did not significantly ($P < 0.05$) enhance the pathogenicity (including carcinogenicity) of fly ash in this animal model.

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PREFACE

JUSTIFICATION AND RATIONALE FOR THE PROJECT

Nickel and its Compounds as an Environmental Health Hazard

The implication of inhaled nickel as an environmental health hazard in man originated mainly from results of several older clinical and epidemiological studies in Germany (Baader, 1937), Great Britain (Barnett, 1949; Doll, 1958; Goldblatt, 1958; Williams, 1958), and Norway (Løken, 1950). Doll (1958), for example, found the death rate from lung cancer in British nickel workers to be approximately five times that of the general population while the death rate from cancer of the nose was as much as 150 times higher. In more recent studies Saknyn and Shabynina (1970) in the USSR reported that the mortality from lung cancer in nickel concentration and smelting plant workers was 2.8 times higher than among the urban population. Tatarskaya (1965) describes two cases of cancer in the nasal area of nickel workers who, contrary to most cases reported in the literature, were not exposed to nickel carbonyl, $Ni(CO)_4$, but to other forms of nickel.

A number of studies with laboratory animals support the aforementioned clinical and epidemiological findings. Gilman (1962), for example, administered nickel oxide and sulfide by means of single intramuscular injections to rats and mice. He found the sulfide to be significantly more carcinogenic in rats than the oxide. In the mice the response to Ni compounds was much lower. Gilman noted a specificity of Ni in several Ni compounds for striated muscle tumorigenesis. These findings were subsequently confirmed by additional studies (Noble and Capstick, 1963; Gilman, et al., 1966). Hueper (1955) administered powdered Ni intrafemorally and subcutaneously to rats and observed sarcomas in 28% of the animals. In later work (Hueper, 1958), he exposed rats and guinea pigs to a powdered Ni aerosol of respirable particle size (concentration $\sim$15 μg/ℓ) 6 hours/day, 4 to 5 days/week for up to 21 months. He observed multicentric adenomatoid formations of alveoli and hyperplastic proliferations of terminal bronchiolar epithelium. Heath and Daniel (1964) injected powdered metallic nickel intramuscularly into rats and produced tumors of striated muscle origin. Ehrlich and Fenters (1972) found increased mortality in some hamster groups challenged with Klebsiella pneumoniae and influenza virus, respectively, in combination with different quantities of NiO administered by intratracheal instillation. Furst and Schlauder (1973), investigating the carcinogenesis of various metals, found that "cadmium and nickel powders, as well as several compounds of these elements, are extremely potent carcinogens in several species." In a table "Epidemiological and Experimental Evidence of Carcinogenicity of Industrial Factors" Wynder and Hoffmann (1972) list nickel as an established carcinogen. Payne (1964), having implanted nine nickel compounds of varying

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solubility in rats, found that the carcinogenicity of the compounds was a function of their solubility and the valence state of the nickel. Inhalation of nickel sulfide by rats resulted in a significantly higher incidence of pulmonary hyperplastic and neoplastic lesions (Ottolenghi et al., 1974).

However, not all investigators were able to demonstrate a carcinogenic action of nickel or certain of its compounds. Saffiotti et al. (1963) investigated the effect of Ni and Co dusts, administered by intratracheal injection in hamsters, and found that "a remarkable hyperplasia of the bronchial epithelium is rapidly induced by cobalt oxide." No mention was made of the effect, if any, of nickel. Wehner et al. (1975a) exposed hamsters to NiO aerosol (mean aerosol concentration = 53 μg NiO/ℓ) 7 hours/day, 5 days/week for the life span of the animals. While the hamsters developed pneumoconiosis as a function of exposure time, leading eventually to severe consolidation of the lungs, no significant carcinogenic effect was found.

Probably the most toxic form of nickel is $Ni(CO)_4$ which is formed when reactive (i.e. finely divided) nickel or its compounds come in contact with CO. $Ni(CO)_4$ is widely used commercially and is one of the most toxic substances found in industrial operations. The threshold limit value (TLV) for a workday has been set by the American Conference of Governmental Industrial Hygienists (1968) at 1 part per billion. By comparison, the TLV for HCN is 10 parts per million, i.e. 10,000 times higher. By further comparison, the TLV for "nickel, metal and soluble compounds" is 1 μg/ℓ. No reference is made to the insoluble compounds. The American Industrial Hygiene Association Hygiene Guide Series pamphlet for nickel mentions this value as "recommended maximal atmospheric concentration (8 hours)" and states that no data are available on the short exposure tolerance for Ni and the atmospheric concentration immediately hazardous to life.

The pathogenicity of $Ni(CO)_4$ has been studied by a number of investigators in rats, guinea pigs, rabbits, cats, dogs, and man (Sunderman, 1966, 1970; Sunderman and Kincaid, 1954; Sunderman et al., 1959; Sunderman et al., 1961; Sunderman and Donnelly, 1965; Armit, 1907; Armit, 1908; Armor, 1932; Hackett and Sunderman, 1967; Kincaid et al., 1953; Bayer, 1939; Brandes, 1934; Krafft, 1939; Mott, 1907). Sunderman and co-workers (1959, 1965, 1966), for example, reported pulmonary carcinomas in rats, following inhalation of $Ni(CO)_4$. Hackett and Sunderman (1967) have shown that intravenous, intraperitoneal and subcutaneous injections of $Ni(CO)_4$ in rats deduce lesions which are practically indistinguishable from those which develop after inhalation. The latter findings indicate that the lung is the target tissue for $Ni(CO)_4$ regardless of its route of administration. Zhdaneeva (1970) claims that the principal toxic effect of $Ni(CO)_4$ is a "disturbance" of the lung capillaries and arterioles, and that acute poisoning does not lead to permanent lesions in healthy individuals.

Sunderman (1970) summarized the toxicity of nickel and its compounds as shown in Table 1. Furst and Radding (1980) provided a recent update on nickel carcinogenesis.

Since, with the exception of contact dermatitis, most nickel-related lesions are caused by inhalation exposure of people (mostly workers in the

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TABLE 1. TOXICITY OF NICKEL AND ITS COMPOUNDS*

Nickel	
Colloidal (Intravenous--Dogs)	LD = 10 to 20 mg/kg
Powdered (Acute Oral--Dogs)	Tolerated: 1 to 3 g/kg
Nickel Salts (Cl, NO_3, SO_4, O)	
Intravenous--Dogs	LD = 10 to 20 mg/kg
Subcutaneous--Rabbits	LD = 1.3 g/kg
Acute Oral--Rats	LD_{50} = 2.0 g/kg
Chronic Oral--Cats	Tolerated: 25 mg/kg/day for 200 days
Nickel Carbonyl--$Ni(CO)_4$	
Mice	LD_{50} = 0.067 mg/ℓ for 30 min.
Rats	LD_{50} = 0.24 mg/ℓ for 30 min.
Nickel Skin Sensitivity	1:10,000 solution of $NiCl_2$ may evoke dermal sensitivity.

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* Table by F. W. Sunderman: Nickel Poisoning, pp. 387-396. In: F. W. Sunderman and F. W. Sunderman, Jr. (eds) *Laboratory Diagnosis of Diseases Caused by Toxic Agents*. Warren H. Green, Inc., St. Louis, Missouri, 1970.

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nickel industry) to nickel or its compounds, the following section summarizes the significance of the problems, and potential problems, created by nickel as an air pollutant.

Nickel in Ambient Air

Occupational Exposures--

Occupational exposures to nickel potentially can occur in mine, smelter, and manufacturing operations, as well as in its use. The sole nickel mine in the U.S. is in Riddle, Oregon, where about 75 people work in open pit mines and 265 people in the smelter (Reno, H.T., U.S. Bureau of Mines, personal communication, 1974). Nickel occurs there in laterite, an ore containing ferric and aluminum hydroxide, some silicates, and nickel oxide. According to statistics published by the U.S. Bureau of Mines (1973) for 1970, Canada ranked as the world's principal nickel producer. Canadian nickel occurs in Ontario, Manitoba, Quebec, Newfoundland, British Columbia, the Yukon, and Northwest Territories as the sulfide in the presence of iron sulfide.

Exposure to nickel carbonyl, formed from nickel matte in the Mond process, may occur in extracting the metal. Exposure to nickel oxide may occur during smelting.

Other occupational exposures occur in industries where nickel is used, for example in corrosion resistant metals such as stainless steel, Ivar, Monel, Inconel, and the Hastelloys; in special alloys--coinage, armor plate, nichrome, permalloy, constantan, and alnico magnets; in ceramics; in Edison storage batteries; in nickel plating; and in catalysts.

Forms of nickel of health concern are the finely divided metal, the sulfide, the oxide, the sulfate and other salts, and a number of nickel complexes.

General Population Exposures--

Comprehensive data available on nickel in ambient air to which the public at large is exposed are contained in the Environmental Protection Agency's (EPA) report (1973) on 1968 and 1969 levels. Nickel concentrations in urban areas were markedly higher than in rural areas where they were mostly below detection limits. Large northern cities tended to have higher Ni concentrations than other cities. New York City ranked first in 1969 with a yearly average of 0.17 μg Ni/m^3 ($\sim 7 \cdot 10^{-9}$ mole ratio). Other northern cities show similar seasonal variation. Southern cities had lower nickel concentrations and showed much less seasonal variation, corresponding to seasonal changes in fossil fuels combustion which is the main source of nickel-polluted air to which the general population is exposed. Although nickel concentrations in ambient urban air are low, human exposures occur for lifetimes.

Source and Change of Population Exposure--

Of the three fossil fuels, coal, oil, and natural gas, only the first two contain significant quantities of nickel. Whether the source of nickel in ambient metropolitan air is coal or fuel oil, or a combination of the two, may be gleaned by comparing ratios of nickel to its analog cobalt in metropolitan air, coal, and oil. EPA's 1969 figures for New York City air yield a Ni/Co ratio of about 20. Air in the steel smelting zone in East Chicago, Indiana, contained little more than half the nickel in New York air, suggesting that smelting did not contribute significantly to the concentration of nickel in the air, but the East Chicago air had a Ni/Co ratio of about 20. From Bertine's and Goldberg's (1971) list of average contents of elements in U.S. coals and oils, average Ni/Co ratios are 3 for coal and 50 for oil, which bracket the observed ratio of 20. The source of nickel in metropolitan air is thus deduced to be a combination of coal and oil combustion.

That human exposure to nickel in metropolitan air is likely to increase appears inevitable because U.S. energy consumption has been increasing more than 4% per year since 1960, and nearly 5% per year during 1972 and 1973 (Dept. of Interior, 1974). Since recent experience shows that national economic health is dependent on energy availability, increased coal use is widely predicted to decrease anticipated energy shortfalls and dependence on energy imports. The Department of Interior's estimate for 1973 coal combustion was 569 million tons, up 8.2% from 1972. The coal industry's goal for 1974 was to produce 660 million tons (Coal Age, 1974), a 16% increase over 1973. Such annual increases cannot be maintained; the maximum growth rate considered maintainable is 10% (Rasmussen, D. E., Battelle-Northwest, personal communication, 1974).

Since fossil fuel combustion evidently is the major source of atmospheric nickel, its concentrations in air may be projected on the basis of energy source mixes anticipated for the future. Of the many energy scenarios available, one by Felix (1974) is used here to obtain average annual growth anticipated for coal (6.7%) and oil (0.56%) through 1985.

EPA (1973) estimated that in 1970 the U.S. injected into the atmosphere 5.7 million tons of fly ash from the combustion of 517 million tons of coal (U.S. Bureau of Mines, 1970). Elemental analyses of ash from 827 U.S. coals by the Bureau of Mines (Abernathy et al., 1969) indicated average values of 0.005% (50 ppm) for ash from Western coals to nearly 0.03% (300 ppm) for ash from Midwestern and Eastern coals. Assuming fly ash is approximately the same, the apparent average emission factor therefore is 55-330 tons nickel per 100 million tons of 1970 coal*.

Average nickel in oil was reported at 10 ppm (Bertine and Goldberg, 1971). Since a 42-gallon barrel of oil weighs about 310 lbs., one billion barrels of oil should contain 3.1 million lbs. or about 1.6 thousand tons of nickel. Of the 6.3 billion barrels of oil consumed in 1973, 5.6 billion barrels were used for fuel and power (Dept. of Interior, 1974). Since nearly 60% of the 5.6 billion barrels were converted to gasoline before combustion, which should have removed much of the nickel, and assuming some

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* $\frac{\text{(50 tons Ni/M tons FA)} \times \text{5.7 M tons FA}}{\text{5.2 hundred M tons coal}}$ = 55 tons Ni per hundred M tons coal.

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fly ash control for oil furnaces, an estimated emission factor of nickel from oil combustion of 500 tons per billion barrels of oil consumed for fuel and power is used here.

Based on figures above for coal and oil combustion and nickel emission factors, Table 2 was prepared to project atmospheric nickel. Other sources of ambient nickel, such as from its use in partial hydrogenation of vegetable oil, are believed minor. Numbers in the table are projections based on estimates; it would not be justified to assume accuracy better than 20% for nickel values. It is doubtful that improved fly ash control technology would have a significant impact before 1980. Thus whatever risk to public health nickel imposed in 1974 (i.e., the time when our original proposal for this study was submitted to EPA), is likely to be nearly 40% greater in 1980.

Nickel in Fly Ash--

Natusch and Wallace (1974) and Campbell et al. (1978) have shown that the nickel content of coal fly ash increases with decreasing particle diameter. That concentrations of nickel in respirable particles are greater than that found in gross ash has also been shown by Fisher et al. (1978a).

Exposure of monkeys and guinea pigs to fly ash aerosol (0.5 μg/ℓ) and SO_2 for up to 78 weeks produced no lesions (Alarie et al., 1973). MacFarland et al. (1971) exposed cynamolgus monkeys to fly ash (0.16 μg/ℓ and 0.46 μg/ℓ, 23 hrs/day, 7 days/week, for 18 months) and found no effects on lung function, hematological and biochemical parameters. Histologically, pulmonary reaction was "similar to that seen with any 'nuisance' dust and is not considered to be evidence of permanent pathological alteration." If lesions had been observed it would have been impossible to attribute them to any one element since 64 minor and trace elements have been identified in coal fly ash (U.S. Bureau of Mines, 1973).

Because of its known toxicity and presence in coal fly ash, it was desirable to investigate whether nickel in fly ash is an important etiological factor. To do so for coal fly ash was the purpose of this research project.

The problem was then to devise a dust which was like fly ash in all respects except that its nickel content was high compared to nickel ordinarily present in fly ash. Thus, if nickel in fly ash causes lung lesions, the increase in nickel should hasten pathologic response in experimental animals exposed to such a dust, and a good case would be established for implicating nickel as one potential cause of pulmonary disease among human populations exposed to fly ash.

A unique approach was taken. Instead of normal fly ash or known nickel compounds, nickel-enriched fly ash (NEFA) was used. It was prepared by mixing nickel acetate into pulverized coal before combustion in a special fossil fuel furnace. A normal fly ash (FA) control, an inherent part of the experiment, was identically prepared from the same coal, but without the addition of nickel. Separate groups of animals were then exposed to aerosols of NEFA and FA. Thus, any difference in response observed can be

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TABLE 2. PROJECTIONS OF ATMOSPHERIC NICKEL FROM COAL AND OIL COMBUSTION

Year[a]	1973[b]	1974	1975	1977	1980	1985
Coal burned (10^8 tons) [c]	5.7	6.1	6.5	7.4	9.0	12.4
Atm. Ni from coal (10^3 tons)	0.31-1.9	0.31-2.0	0.36-2.1	0.41-2.4	0.50-3.0	0.66-4.1
Oil burned (10^9 barrels)[c]	5.6	5.6	6.7	5.7	5.8	6.0
Atm. Ni from oil (10^3 tons)	2.8	2.8	2.8	2.9	2.9	3.0
Total atmospheric nickel (10^3 tons)	3.1-4.7	3.1-4.8	3.2-4.9	3.3-5.3	3.4-5.9	3.7-7.1

(a) Some years omitted to save space.

(b) Estimate for coal and oil combustion (Dept. of Interior, News Release, March 14, 1974).

(c) Annual growth rates: coal, 6.7%; oil, 0.56% (Felix, F.: Energy Independence: Goal for the '80s. Electrical World, 52-55, March 1, 1974).

ascribed to nickel. Lack of lesion development reported in earlier inhalation studies with fly ash (MacFarland et al., 1971; Alarie et al., 1973) could then be explained by insufficient aerosol concentrations and exposure times, because at the low concentration of nickel in normal fly ash it might take years to evoke pathological response if nickel were, indeed, responsible for such a response.

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ABSTRACT

The objective of this research program was to determine biological effects of inhaled NEFA (nickel-enriched fly ash) compared to FA (regular fly ash) as a function of aerosol concentration and cumulative exposure time.

Considerations leading to the enrichment of FA with nickel, the enrichment procedures, and acute and subchronic exposures of hamsters to NEFA and FA are described.

Groups of 102 male Syrian Golden hamsters were chronically exposed to ~70 μg/ℓ respirable NEFA aerosol (high NEFA group), ~17 μg/ℓ (low NEFA group), or ~70 μg/ℓ FA 6 hrs/day, 5 days/week, for up to 20 months. An identical control group received sham exposures. The NEFA particles of respirable size contained approximately 6% nickel, compared to about 0.3% for FA. Five hamsters/group were sacrificed after 4, 8, 12 or 16 months of exposure. An additional 5 hamsters/group were withdrawn from exposure at the same intervals for lifelong observations.

Exposures to NEFA had no significant effect on the apparent well-being, body weight and life span of the animals although heavy deposits of NEFA in the lungs were demonstrated. The lung weights of the high NEFA- and of the FA-exposed animals, however, were significantly ($P < 0.01$) higher than those of the low NEFA group and the controls. There was no significant difference between the MBWs of the high NEFA group and the FA group. The mean lung volumes were significantly ($P < 0.01$) larger for the high NEFA group and the FA group than for the low NEFA group and the controls.

There was a 100% incidence of dust deposition, referred to in this report as anthracosis, in the lungs of exposed hamsters. Incidence and severity of interstitial reaction and bronchiolization were significantly higher in the dust-exposed groups than in the sham-exposed controls. The severity of anthracosis, interstitial reaction and bronchiolization was significantly lower ($P < 0.01$) in the low NEFA group than in the high NEFA and FA groups. This dose-effect relationship reflects the two different dose groups, namely low NEFA versus high NEFA and FA. While two malignant primary thorax tumors were found in two hamsters of the high NEFA group, no statistically significant carcinogenesis was observed. Of the exposure-related changes, only anthracosis decreased as a function of recovery time.

Comparison of pulmonary nickel burdens after 20 months of exposure with the aerosol concentration, suggests that the pulmonary clearance rate was slower in the high NEFA exposure group than in the low NEFA exposure group.

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The results of our study lead to the conclusion that the addition of nickel to fly ash under our experimental conditions did not significantly ($P < 0.05$) enhance the pathogenicity (including carcinogenicity) of fly ash in our animal model.

This report was submitted in fulfillment of Contract #68-03-2457 by Battelle Pacific Northwest Laboratories under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period from November 10, 1976 through December 31, 1979. Work on the report was completed as of February 22, 1980.

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LIST OF ABBREVIATIONS

AED = Aerodynamic equivalent diameter
AMAD = Activity median aerodynamic diameter
BCL = Battelle Columbus Laboratories
BNW = Battelle Pacific Northwest Laboratories
EPA = United States Environmental Protection Agency
FA = Fly ash
FAAS = Flame atomic absorption spectrometry
GI = Gastro-intestinal
GSD = Geometric standard deviation
H&E = Hematoxylin and eosin
LSL-II = Life Sciences Laboratory II
MBW = Mean body weight
MMAD = Mass median aerodynamic diameter
NEFA = Nickel-enriched fly ash
SD = Standard deviation
SEM = Standard error of the mean;
Scanning electron microscope
SNOP = Systematized Nomenclature of Pathology
TLV = Threshold limit value
WDFM = Wright Dust Feed Mechanism

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The names and responsibilities of staff members having contributed significantly to the work described in this report are listed below.

Dr. Alfred P. Wehner, project management and principal investigator assisted by Edward M. Milliman, animal exposures; Dr. Gerald E. Dagle, pathology assisted by James F. McShane, necropsy; Dr. Owen R. Moss, assisted by Edward M. Milliman, NEFA and FA aerosol generation and characterization; M. Gilbert Brown, animal care; Richard E. Barrett, Battelle Columbus Laboratories, FA generation and enrichment of FA with nickel; Dr. Roger E. Schirmer, lung tissue analyses for Ni; Donald L. Stevens, Jr. and Judith A. Mahaffey, statistics; Drs. William C. Cannon and Owen R. Moss, preparing the aerosol exposure to neutron-activated fly ash; and Connie L. Wilkerson, neutron activation of FA and γ-ray analysis of samples.

Edward M. Milliman, Barbara D. Holloway, and Deborah L. Walton provided technical assistance in exposing the animals to NEFA and FA aerosols. Edwin F. Blanton provided technical assistance in exposing the animals to neutron-activated FA. James H. Reeves handled the reduction of the neutron activation experimental data by computer analysis; Lavern L. Baker prepared and counted the samples.

Dr. Richard A. Pelroy conducted the Ames Bioassays.

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INTRODUCTION

Battelle Pacific Northwest Laboratories (BNW) submitted the original proposal for this project in May, 1974. Subsequently, a revised scope of work was approved by EPA and authorization to start work was received in November, 1976.

The project encompassed three phases, namely a preparatory phase, the acute and subchronic exposures phase, and the chronic exposure phase. In addition, a pulmonary deposition, translocation and clearance experiment with neutron-activated fly ash was conducted parallel to the chronic exposures to provide information on the fate of the fly ash deposited in the lungs of the exposed hamsters.

During the preparatory phase, suitable coal was procured by Battelle Columbus Laboratories (BCL) for the generation of FA and NEFA at BCL, while facilities and equipment for the animal exposures were prepared at BNW.

The objectives of the acute and subchronic exposures were to determine a) the toxicity of FA and NEFA under the conditions of the acute and subchronic exposures and b) the optimal dose levels for the chronic exposures. For inhalation studies, the exposure dose level is defined as the product of the respirable aerosol concentration and the exposure time. It should be noted that this parameter refers to the dose offered to the exposed animals, but not to that fraction of the offered dose that was deposited and retained in the lungs. Depending on the nature of the inhaled material, generally less than 10% of the inhaled material is deposited in the lungs. Optimal dose levels for chronic exposures are defined as those levels high enough to eventually produce lesions (e.g., cancer) due to chronic exposure to a given agent or combination of agents, yet not so high as to produce acute or subchronic toxicity. Acute or subchronic toxicity can interfere with the objectives of chronic exposures by causing intercurrent pathologic conditions and perhaps premature death of the exposed animals.

The objective of the chronic exposures was to determine under controlled laboratory conditions the biological effects of prolonged inhalation of NEFA and FA in our animal model, the Syrian Golden hamster. More specifically, the experiments were designed to investigate:

- the development of NEFA- and FA-induced biological effects as a function of aerosol concentration and cumulative exposure time,
- mortality as a function of aerosol concentration and cumulative exposure time,

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- reversibility of NEFA- and FA-induced lesions as a function of aerosol concentration, cumulative exposure time, recuperation (observation) time and age, as groups of hamsters were periodically withdrawn from the aerosol exposures and maintained for observation for the remainder of their natural life span up to the age of 22 months.

Criteria for evaluating biological effects were body weight, mortality, and gross and histopathologic findings.

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CONCLUSIONS

Exposures to NEFA had no significant effect on the apparent well-being, body weight and life span of the animals although heavy deposits of NEFA in the lungs were demonstrated. The lung weights of the high NEFA- and of the FA-exposed animals, however, were significantly ($P < 0.01$) higher than those of the low NEFA group and the controls and might be a sensitive indicator of early lung changes. There was no significant difference between the MBWs of the high NEFA group and the FA group. The mean lung volumes were significantly ($P < 0.01$) larger for the high NEFA group and the FA group than for the low NEFA group and the controls.

There was a 100% incidence of dust deposition, referred to in this report as anthracosis, in the lungs of exposed hamsters. Incidence and severity of interstitial reaction and bronchiolization were significantly higher in the dust-exposed groups than in the sham-exposed controls. The severity of anthracosis, interstitial reaction and bronchiolization was significantly lower ($P < 0.01$) in the low NEFA group than in the high NEFA and FA groups. This dose-effect relationship reflects the two different dose groups, namely low NEFA versus high NEFA and FA. While two malignant primary thorax tumors were found in two hamsters of the high NEFA group, no statistically significant carcinogenesis was observed. Of the exposure-related changes, only anthracosis decreased as a function of recovery time.

The results of our study thus lead to the conclusion that the addition of nickel to fly ash under our experimental conditions did not significantly ($P < 0.05$) enhance the pathogenicity (including carcinogenicity) of fly ash in our animal model.

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MATERIALS AND METHODS (GENERAL*)

PREPARATION OF REGULAR AND NICKEL-ENRICHED FLY ASH

The Multifuel Furnace

BCL's multifuel furnace was designed to generate flue gas and FA under conditions closely simulating those of a power-generating station. This implies combustion at a sufficiently high temperature with a proper cooling schedule to produce flue gas and FA having physicochemical properties similar to those of a typical central station boiler and its associated stack and plume. In addition, the laboratory-scale system used in this program was flexible enough to permit firing with either pulverized coal or residual oil.

The multifuel furnace facility is usually operated with an electrostatic precipitator for coal firing, but without the electrostatic precipitator in firing oils. The time-temperature profiles in the other sections of the facility can be adjusted for the inclusion or exclusion of the precipitator.

Figure 1 is a schematic diagram of the gas-combustion and flue-gas-conditioning system with major sections indicated. The major sections of the facility are discussed below.

Figure 2 is a photograph of BCL's multifuel furnace used in this program. This small-scale furnace consists of a cylindrical combustion chamber approximately 40 cm in diameter by 2.3 m in length. The furnace is lined with three layers of firebrick and insulation to accommodate surface temperatures up to 1600°C. At the outlet, the diameter of the furnace is reduced to 13 cm to enclose the flame, provide for normal recirculation, limit radiation losses, and provide sufficient gas velocity to keep FA suspended in the gas stream. Viewports along the axis of the furnace provide for visual inspection during periods of adjustment of firing conditions.

During normal operation of the furnace, natural gas is fired to maintain system temperatures at approximately the desired levels on a more or less continuous basis between tests. Upon switching to coal firing, the entire system is allowed to equilibrate for several hours before any data are collected.

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*Materials and methods specific to the acute, subchronic and chronic experiments and the pulmonary deposition, translocation and clearance experiment, are outlined in the appropriate sections describing these experiments.

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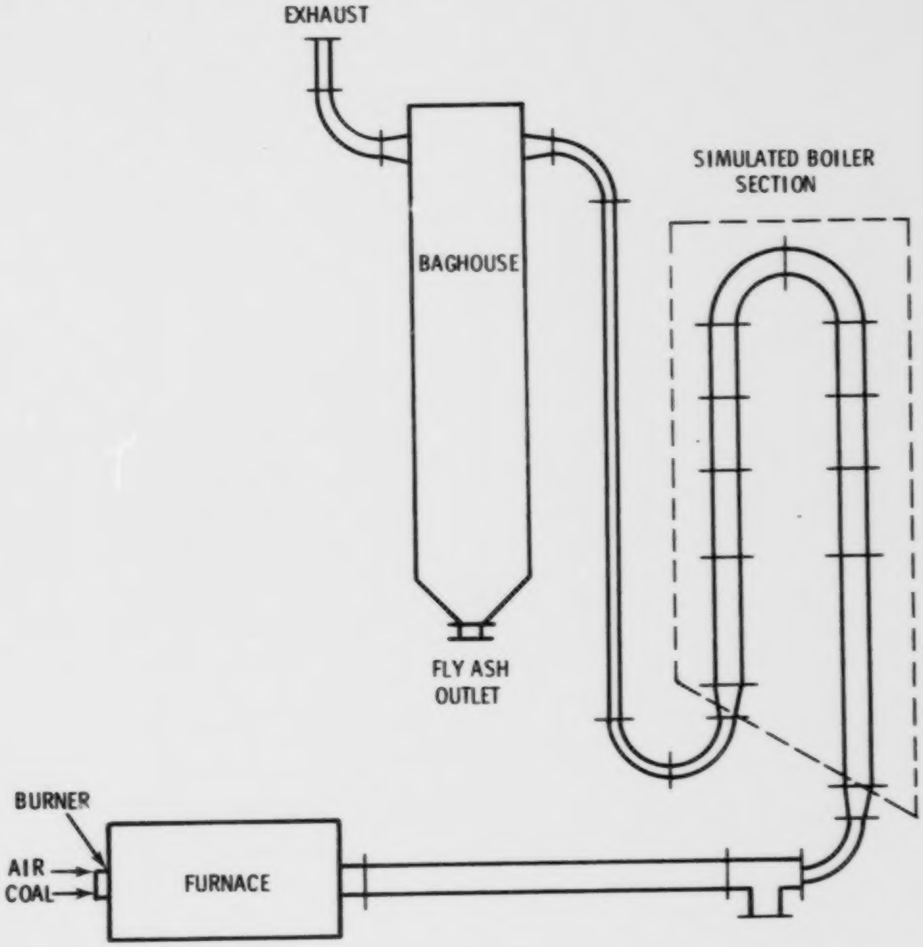

FIGURE 1. Schematic of fly ash laboratory system.

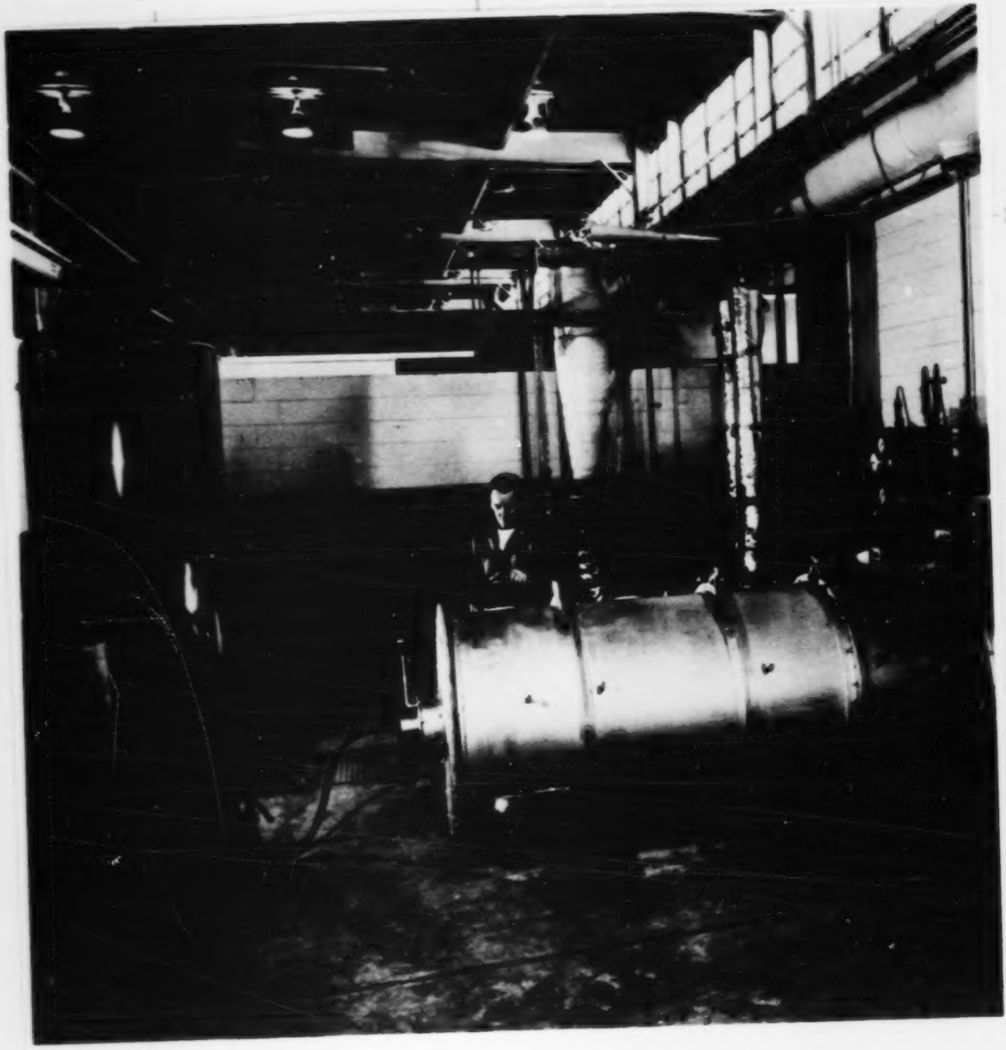

FIGURE 2. Coal feeder and Battelle multifuel furnace.

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With coal firing, a dispersion of the fuel in air is fed to the burner (at a rate of 14 to 18 kg/hr) via a screw feeder mounted within a pressurized coal hopper. The multifuel furnace is capable of being fired with coal at up to about 18 kg/hr. Assuming 10% ash in the coal, ash is released at a rate of about 1.8 kg/hr. About 90% of the ash is deposited as slag in the furnace and exhaust duct. Thus, about 10% of the ash, i.e., about 180 g/hr, pass through the remaining ducting. Five hours of successful FA collection resulted in the collection of about 900 g of FA per day.

Simulated Boiler-Economizer Section

The simulated boiler-economizer section of the rig is constructed of stainless steel pipe lined with a castable refractory material and insulated on the outside. Gas velocities in horizontal portions of this section are typically 18 m/sec, and velocities in vertical portions are about 1.5 m/sec. Gas temperature decreases from about 1425°C at the inlet to about 200°C or less at the outlet.

Electrostatic Precipitator or Baghouse Collector

An electrostatic precipitator has been used for removal of FA between the boiler and stack sections of the system when coal was fired. Typically, the inlet and outlet temperatures of the precipitator were 190°C and 150°C, respectively.

To collect respirable FA, it was deemed advantageous to replace the electrostatic precipitator with a baghouse-type collector. The electrostatic precipitator has a measured efficiency of up to about 80% while a good baghouse installation generally collects 98% or more of the FA. No other facility modifications were made.

Coal

The theory that nickel occurs in FA as nickel-iron spinels (see below) led to the suggestion that the coal for NEFA production be a high-iron coal so that the largest possible quantity of nickel could be added to a given quantity of coal. The possibility that nickel may occur in some other form (e.g., in glasses) did not detract from the selection of a high-iron coal.

BCL staff reviewed available coals with coal and boiler company representatives and determined that some Ohio and Indiana coals have relatively high (~3%) iron contents. Specifically, the Pittsburgh No. 8 seam of Ohio coal was identified as a seam of high-iron coal. Because most of the iron in coal occurs as iron sulfide (pyrites), high-iron coals are also high-sulfur coals. Most coal mining companies having worked in the Pittsburgh No. 8 seam were either closed down or mining other seams because of the low demand for high-sulfur coal. One company mining this seam was finally located and 6 tons of the desired coal were obtained.

Form of Nickel for Enrichment of Fly Ash

Information on the form of nickel in coal and/or FA would have been helpful for selecting the most appropriate nickel compound for addition to the coal for generating NEFA. In our proposal dated May 16, 1974, we stated on page 15, "Nickel appears to be in coal fly ash as a spinel-type complex containing oxides of nickel, iron, silicon, and aluminum, along with many other elements." It was further suggested that the nickel spinel in the FA might be Ni Fe_2O_4. These statements were based on a personal communication (reference 49 in our proposal) with M. D. Schlesinger, then U.S. Bureau of Mines (subsequently ERDA).

Since nickel occurs naturally only at low concentrations in coal (average in US coals ∼100 ppm), and thus also in FA, identification of nickel compounds is rather difficult. It was therefore of interest to determine whether the statements on the form of nickel in fly ash were based on analytical data or on opinion. Apparently there were no reports on analyses of nickel compounds in either coal or FA.

Based on work with cobalt at Grumman (Tobin et al., 1973) and the $NiO-Al_2O_3-SiO_2$ phase diagram (American Ceramics Society, 1969), it was suggested that nickel in coal may be in a $NiO \cdot Al_2O_3$ spinel form. On the other hand, the nickel might be present as $NiFe_aSi_bAl_cO_d$ glasses, rather than as well structured crystals.

In light of the above information, it appeared unlikely that nickel in FA occurs as straight nickel-iron spinels. The presence of relatively abundant quantities of silicon and aluminum (and all the eutectics that this permits) strongly suggests that nickel is most likely present in the glass form. If this is true, it probably would not be too important in which form nickel would be added to the coal.

If nickel occurs in the ash as a complex nickel-iron spinel, the best nickel additive would have been a complex nickel-iron sulfide. Given the short residence time in a boiler furnace or in BCL's multifuel furnace, it is unlikely that spinels would form from simple iron and nickel compounds. Unfortunately, complex nickel-iron sulfides do not appear to be commercially available. A potential source of complex nickel sulfides might have been nickel ores, such as are mined near Sudbury, Canada. However, these ores contain significant quantities of other elements (e.g., copper), which could have influenced the animal studies. Had it been desired, nickel-iron sulfides could have been produced at BCL by melting nickel and iron sulfides together in an inert atmosphere.

Since the iron in coal normally exists as iron sulfide (pyrites) and nickel is presumed to also exist in sulfide form (either simple or complex), adding nickel sulfide had some appeal. The nickel sulfide could have been mixed with the raw coal before pulverization, and the pulverization operation could have served to further mix the coal and nickel. During combustion, the nickel would have been oxidized.

Another alternative was to add nickel as nickel acetate. Nickel acetate is highly soluble and could be sprayed on crushed coal. Evaporation of

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the water would leave a coating of nickel acetate on the coal. Pulverization of the nickel-coated coal would serve to mix intimately the nickel and coal. Alternately, if nickel acetate could be acquired in a pelletized form, it could be added like nickel sulfide (discussed above). In either case, nickel acetate readily decomposes and the combustion process would produce nickel oxide.

Test Firing of Coal Sample

It was therefore decided to make an attempt at determining the form in which nickel exists in FA by test-firing a small quantity of coal in BCL's multifuel furnace and by collecting and analyzing the FA from this test run. If the analyses could identify the form in which nickel occurs in the FA, the nickel compound most suitable for the enrichment process could be selected. To avoid scatter of the FA sample over the large surface of the baghouse collector (which would have made recovery difficult), the sample from the flue gas stream was collected using an air-pollution-emission particulate sampling rig.

Particulate loadings of the flue gas for the test firing were on the order of 400 mg/m^3. At the rate at which the furnace was being fired, flue gas was generated at a rate of 150 m^3/hr. This is within the range of earlier estimates.

Particle size measurements during the test firing gave the following results:

18%	<1.0 μm
50%	<3.0 μm
94%	<10.0 μm

Such a particle size distribution was suitable for the chronic inhalation study.

Analysis of Fly Ash for Nickel

Analysis of the aforementioned FA sample, collected from the test firing, showed a nickel content of 250 ppm. This concentration of nickel is well within the typical range for U.S. coals, estimated to be 0 to 700 ppm, and above the estimated U.S. average of about 100 ppm.

The second stage of analysis involved the FA (~9.5 kg) generated and collected for the animal exposures. It comprised an attempt to isolate nickel-rich particles for securing a sample sufficiently rich in nickel to permit nickel compound identification. Examination of the sample by electron microprobe showed that the nickel generally was uniformly distributed through the FA sample. This precluded isolation of local particles of high nickel content and compound identification. Since a large sample of FA might have permitted concentration of nickel-rich particles and thus facilitate nickel compound identification, an effort was made to concentrate the nickel in the FA sample by magnetic techniques. However, nearly the entire sample responded to the magnet and, thus, no concentration was achieved.

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Hence compound analysis was not possible. Since the scope of work did not include the determination of the type of compound in which nickel occurs in FA, no further attempts were made to identify the nickel compound.

The FA sample was then water- and acid-washed to determine the water-soluble and acid-soluble fractions. Results of these analyses were:

total nickel	390 ppm
water-soluble nickel	280 ppm (72% of total Ni) *
acid-soluble nickel	280 ppm (72% of total Ni)

These results suggest that the nickel probably occurs in the form of sulfate or nitrate salts. Determination of the specific compounds would have been expensive, and, as stated above, did not fall within the scope of work.

Choice of Nickel Additive

A review of the above nickel analyses and considerations of how nickel should be added to the coal, led to the conclusion that nickel might be added to the coal as a sulfide (either nickel or nickel-iron sulfide), or as nickel acetate. Adding a sulfide might appear more desirable if the final form of nickel is a sulfate, since the nickel and sulfur are already bound together and only require the addition of oxygen to form the sulfate. However, nickel sulfate decomposes (losing SO_3) at 840°C, well below the 1370 to 1480°C temperatures in the furnace. Thus, forming nickel sulfate too early in the flame may not be especially conducive to obtaining sulfate in the FA. Also, nickel sulfide is not readily available and preparation might have cost $65/kg (several hundred kg would have been needed). It was therefore concluded that nickel acetate would be a more suitable compound than nickel sulfide to enrich the coal with nickel.

Preparation and Analysis of Nickel-Enriched Fly Ash

A test quantity of pulverized coal, enriched with nickel acetate in a ratio of 1 part by weight nickel acetate to 14 parts coal, was fired in the furnace/baghouse system. A portion of the NEFA obtained from firing this coal was analyzed at BCL for total nickel, water-soluble nickel, and acid-soluble nickel. One kilogram of this material was sent to BNW for use in the acute and subchronic animal exposures.

Analysis of 1:14 nickel-enriched fly ash gave the following results:

total nickel	89,500 ppm
water-soluble nickel	24,300ppm (27% of total Ni)
acid-soluble nickel	19,800 ppm (22% of total Ni).

The results show that the ratios of water-soluble and acid-soluble nickel to total nickel were significantly lower in the NEFA than in the baseline FA.

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* NOTE: If certain compounds are both water-soluble and acid-soluble, the cumulative percentage of water-soluble plus acid-soluble fractions may exceed 100%.

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An additional quantity of NEFA was then prepared with coal enriched only about one-third as much as in the first firing, i.e., about 1 part nickel acetate to 40 parts coal, to determine whether the nickel values in the fly ash from the 1:40 nickel-enriched coal will be similar to those in the baseline FA.

The previously conducted dissolution experiments were repeated using longer dissolution times and repeated measurements to determine whether the previously obtained values might be misleading due to a combination of different particle sizes (surface areas) of the two types of fly ash (NEFA and baseline FA) and insufficient dissolution time. The results of the second dissolution experiments are shown in Table 3.

Assuming that the weight percentage is correct for Sample 1a, (total FA dust sample), then the nickel was confined to the 10% of the weight of this dust composed of fine particles (less than 53 μm sieve size). The nickel in the 3% and 9% NEFA was uniformly distributed through the fly ash and dissolved at a rate independent of sieve size. The fraction of the total nickel extracted in 24 hours is approximately the same as that extracted from the less-than-53 μm size fraction of FA. Since the nickel in the respirable fraction of the three FA samples appeared to be extracted at the same rate, the highest (i.e,. 9% Ni) NEFA was decided upon for the animal exposures.

Thus, 8.6 kg of NEFA were prepared by BCL for BNW. This represented the estimated quantity of NEFA required for the chronic exposures. The estimate was based on the assumption that the respirable NEFA aerosol concentration for the chronic exposures would not exceed 50 μg/ℓ, or 1 WDFM* cup/exposure chamber/exposure day.

THE ANIMAL MODEL

The Syrian Golden hamster [*Mesocricetus auratus*, outbred LAK:LVG (SYR) Charles River Lakeview, Wilmington, MA] was chosen as the animal model because of the positive response of this species in terms of lesion developments to various pathogenic agents including carcinogens (Homburger, 1979; Dontenwill, 1970; Montesano and Saffiotti, 1970; Montesano et al., 1970; Dontenwill and Mohr, 1961; Herrold and Dunham, 1963; Mohr et al., 1966; Montesano and Saffiotti, 1968; Mohr, 1970). Only male hamsters were used because there are, to our knowledge, no indications in the literature to suggest significant sex-related differences in the incidence of those lesions that one might expect to develop as a result of exposure to this type of dust. Lesions one might expect to develop under these conditions in the primary target organ, the lung, are primarily pneumoconiosis, probably emphysema, and conceivably cancer. Also conceivable were lesions in potential secondary target organs, e.g., liver and kidneys, due to leaching and translocation of potentially toxic elements from the NEFA and FA in the lung to these organs. Using only one sex for a given number of experimental animals

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* Wright Dust Feed Mechanism (Wright, 1950).

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TABLE 3. SUMMARY OF THE RESULTS OF THE DISSOLUTION EXPERIMENTS

		% of Nickel in Solution			
		*Acid-Soluble Ni		Water-Soluble Ni	
Sample	Total Ni In Sample (wt. %)	1 Hour (80-90°C)	24 Hours (80-90°C)	1 Hour (80-90°C)	24 Hours (80-90°C)
1a. Baseline fly ash (total dust)	0.032	100†	81†	78	94
1b. Baseline fly ash (fine particles only**)	0.38	37	42	32	34
2a. 3% by weight Ni*** (total dust)	4.61	26	28	13	22
2b. 3% by weight Ni*** (fine particles only**)	4.81	32	39	22	27
3a. 9% by weight Ni*** (total dust)	8.64	29	34	22	26
3b. 9% by weight Ni*** (fine particles only**)	7.82	30	32	26	28

---

* Hot, 10-normal nitric acid.

** The sieve size was 270 mesh ($\sim$53 μm).

*** The nickel compound mixed with the coal before firing was nickel acetate.

† The 1-hr values (100%) were higher than the 24-hr values (81%), probably due to variability in the analysis of samples near the limit of detection of the method.

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allows for more powerful statistical tests in the evaluation of the results. Males were chosen because of easier handling characteristics and the absence of estrus cycling.

ANIMAL CARE AND ENVIRONMENTAL HYGIENE

Shipping of the hamsters was scheduled so as to ensure the shortest possible transit time. After arrival at the local airport (Pasco), the animals were transported in our air-conditioned animal van to BNW's animal quarters in the LSL-II building where they were maintained for a mandatory 3-week quarantine period before being released for the experiments.

Serum from six randomly selected hamsters was collected and sent to Microbiological Associates, Bethesda, MD, for murine virus antibody determination. The results are shown in Table 4. Positive titers were found for Sendai virus (SV5) and pneumonia virus of mice (PVM) in these sera. SV5 is not pathogenic for hamsters, and PVM is not likely to cause lesions. Histopathologic findings for lung tissue from all 6 hamsters were negative. Hamsters with netative titers were not commercially available.

A central air conditioning system in LSL-II provided "once-through", filtered (HEPA filter, retaining 99.9% particles >0.3 μm) air. Temperature and relative humidity in the experimental rooms and in the exposure chambers were measured and recorded twice daily, namely shortly after the beginning (about 8:00 a.m.) and shortly before the end (about 2:00 p.m.) of the daily exposures. The animals were maintained in their cages (Figure 3) which were positioned in the exposure chambers on a 12-hour light:12-hour dark cycle. They had free access to Wayne Lab-Blox F4 laboratory animal feed (Allied Mills, Inc., Chicago, IL) and to water. The animals were weighed biweekly during their growth period and monthly thereafter, using a Toledo #8130 digital electronic balance.

The drinking water, supplied to the cages by means of an automatic water system (Systems Engineering, Inc., Napa, CA), came from a city filter plant and from city wells. The filter plant takes water directly from the Columbia River. This water averages about 60 to 80 ppm hardness. The wells are located around a recharge basin. Water is pumped into the recharge basin from the river. The well water has a hardness of about 100 to 200 ppm. The filter plant operates nearly all the time with the wells being used as a supplemental source. During summer, we can expect to get mostly well water. Chlorine is added at the rate of 1.0 to 1.5 ppm. The water is softened to less than 2 ppm hardness by means of an Industrial Water Softener, Model 2R 2240 (Illinois Water Treatment Company, Rockford, IL) before entering the main water distribution lines in our laboratory. The Benton County Health Department routinely cultures the water from various sites along the distribution lines. In our laboratory an 800-ml water sample was collected from the automatic drinking water system and subjected to a standard water test for coliform microorganisms. Less than one organism per 10 ml of water was found. All organisms cultured were nonenteric, nonpathogenic species.

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TABLE 4. MURINE VIRUS ANTIBODY DETERMINATION IN HAMSTER SERUM*

	Hemagglutination Inhibition			Complement Fixation	
Sample Number	PVM	Reo3	SV5	Sendai	LCM
125	10	-	-	40	-
126	10	-	-	80	-
127	10	-	-	40	-
128	40	-	-	160	-
129	20	-	20	40	-
130	10	-	-	80	-
Significant Titer	10	20	20	10	10

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* These tests were performed by Microbiological Associates, Bethesda, MD.

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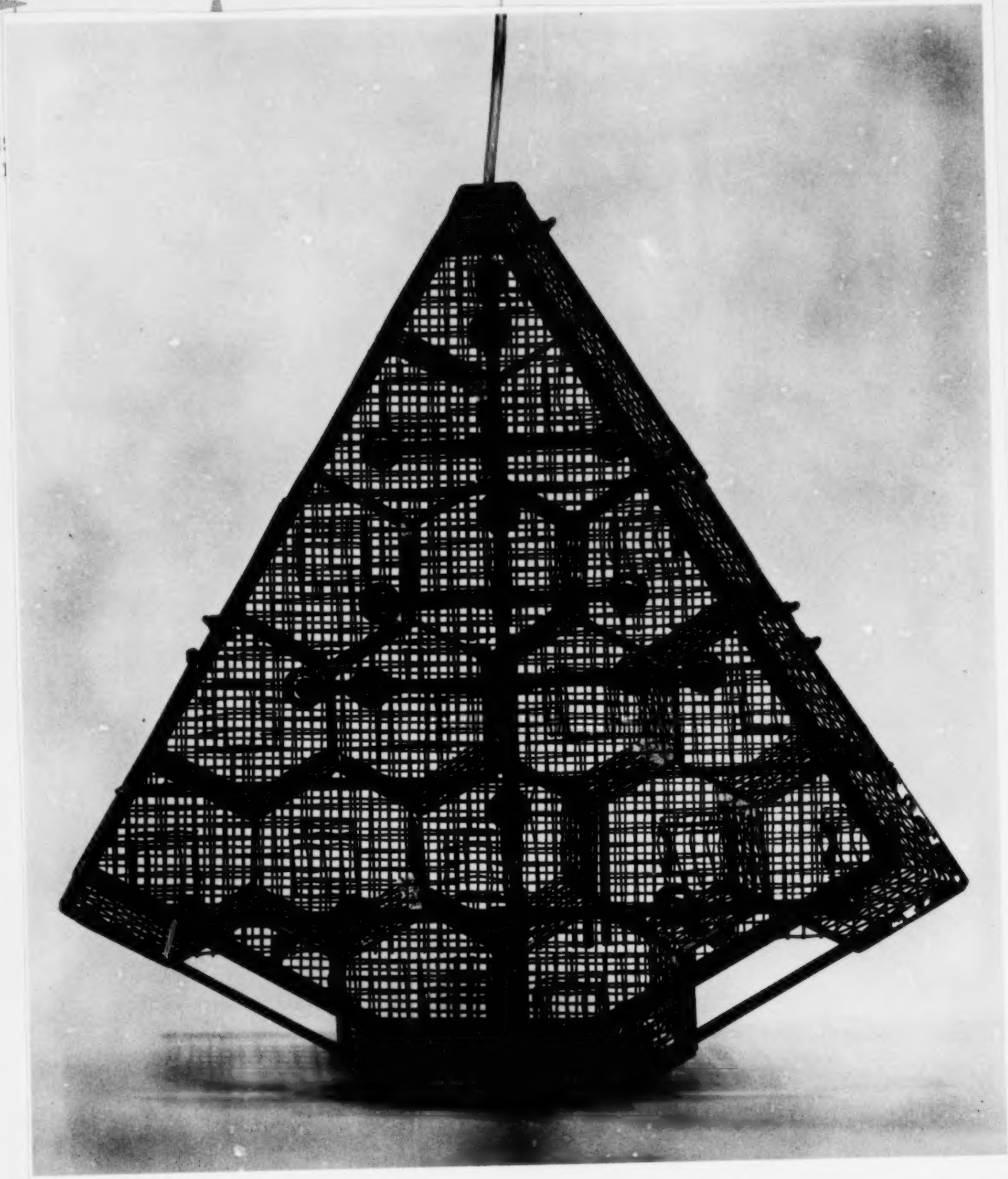

FIGURE 3. Top view of stainless steel wire mesh cage with 17 individual 150-cm^2 compartments and manifold of automatic drinking water system.

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Cages were changed once per week. They were washed in a Better Built Cage Washer Model #6300 (Better Built Machinery Corp., Saddle Brook, NJ). The washing cycle started with a 5-minute wash with hot (∼85°C) water containing Exalt acid (Pennwalt Corp., Philadelphia, PA) at a pH of 1, followed by rinsing with a neutralizer. The racks and cages were then washed for another 5 minutes with hot water containing a detergent (Clorclean, Pennwalt Corp.) at a pH of 10, followed by 3 minutes of rinsing with hot water. Floors were washed daily with Sanikleen (West Chemical Products, Inc., New York, NY) solution. The chambers were rinsed out daily and deep-cleaned weekly with 300 ppm of a quaternary ammonia solution injected into 45°C wash-down water.

THE AEROSOL EXPOSURE SYSTEM

The aerosol exposure system, shown in Figure 4, has been previously described in detail (Wehner et al., 1972). Briefly, a 2700-ℓ hexagonal lucite chamber (1.2 m high x 1.80 m wide between opposing walls) provided for whole-body, single-tier exposures of up to 102 individually caged hamsters. Six sectional stainless steel wire cages (Figure 3) rested on a turntable which provided easy access to the cages through a sealed door. Each of the sectional wire cages had 17 individual compartments. Each compartment had 150 cm^2 of floor space. Walls and top of the cages were made of 2-mesh stainless steel wire; for the cage floor 3-mesh stainless steel were used. The position of each cage in the chamber was rotated from exposure to exposure to randomize any biased conditions that might have existed between different cage locations within the chamber. The aerosol, generated by a WDFM, entered the chamber at the center of the top. The dispersion in the chamber was aided by a small fan near the top. The aerosol left the chamber through flexible exhaust tubing via a filter and a flowmeter and valve.

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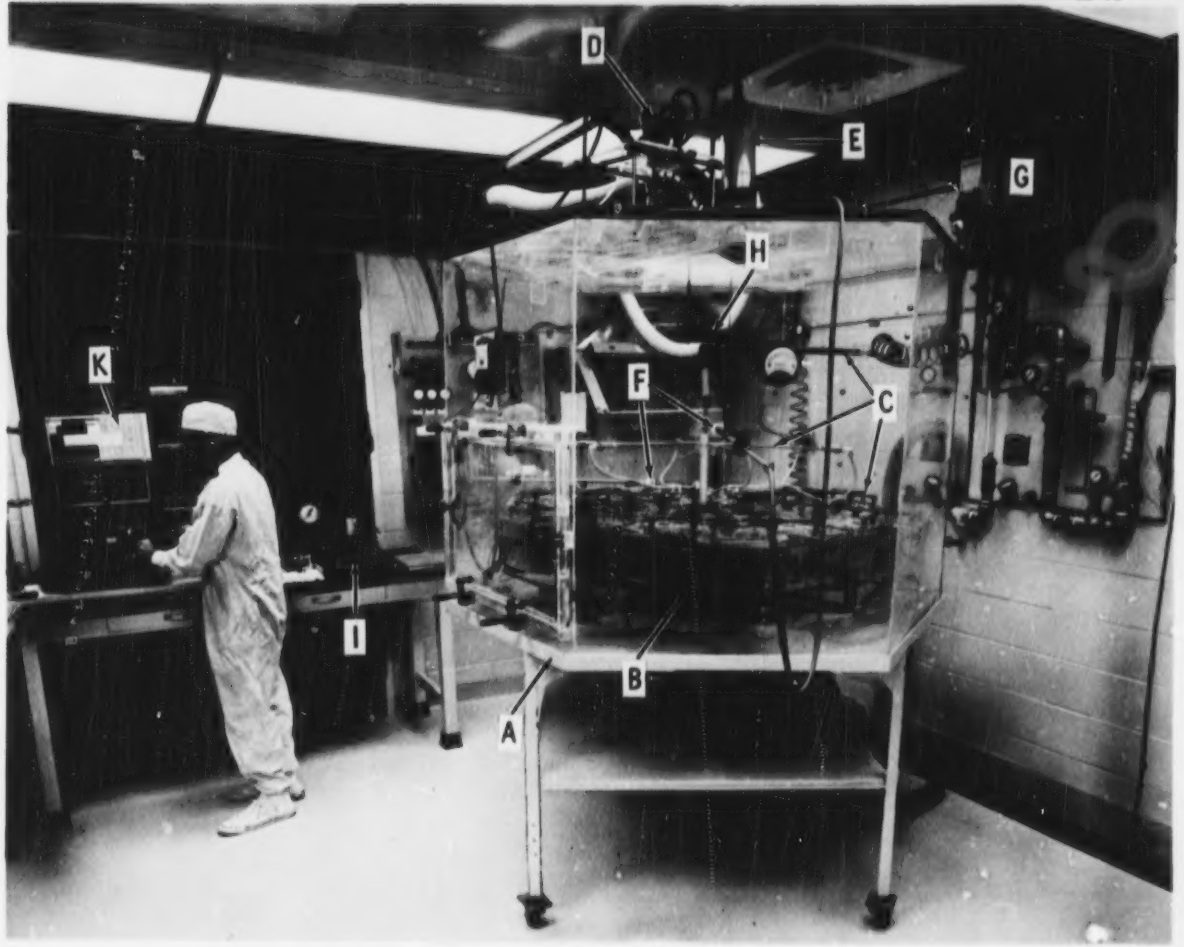

FIGURE 4. Aerosol exposure system. A = Aerosol exposure chamber; B = hamster cages; C = drinking water system; D = Wright dust feed mechanism (WDFM); E = elutriator; F = aerosol sampling probes; G = air drier for providing dry air for aerosol generation by WDFM; H = mixing fan; I = press to pack fly ash into dust container of WDFM; K = microbalance for weighing filter paper samples.

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ACUTE TOXICITY TEST

The objective of the acute toxicity test was to expose a group of hamsters for 1 day (6 hours) to the highest practical concentration of respirable NEFA aerosol to investigate the resultant biological effects and thereby obtain guidelines for determining suitable NEFA aerosol concentrations first for the subchronic (2 months) and ultimately for the chronic exposures.

The toxicity of NEFA was unknown. Sunderman (1959, 1965, 1966) has shown that $Ni(CO)_4$ is highly toxic. Wehner et al. (1975), on the other hand, found no Ni-specific toxicity nor an effect on mortality in hamsters having received life span exposures to the relatively high NiO aerosol concentration of 53 µg/ℓ. Further, Alarie et al. (1973) found no toxic effects in monkeys and guinea pigs exposed to ordinary fly ash aerosols (concentration = 0.5 µg/ℓ) for up to 78 weeks. One could speculate that the toxicity of NEFA might lie somewhere between these two extremes. It was conceivable, though, that its toxicity might be similar or perhaps even lower than that of NiO or FA. In this case it would be difficult to achieve a sufficiently high aerosol concentration for a lethal effect because there are aerosol concentration limits above which the aerosol becomes unstable due to increasing coagulation of the aerosol particles. Unstable aerosols are, of course, unsuitable for controlled scientific experiments. It should be realized in this context that Ni, even in NEFA, accounts for only a small amount of the total aerosol mass. Thus, to obtain a given Ni concentration a very much higher NEFA aerosol concentration would be required. This holds true even more so for the FA aerosol, which was not Ni-enriched. Such aerosols probably would be rather unstable. On the other hand, it was quite possible that NEFA could prove toxic at considerably lower concentrations, thereby permitting the use of stable aerosols. The acute toxicity test was performed to answer these questions.

Seventeen 4-month-old hamsters were exposed for 6 hours to NEFA aerosol. Total mean aerosol concentration was approximately 590 ± 21 (SD) µg/ℓ; the "respirable" fraction was approximately 220 µg/ℓ. This high aerosol concentration was achieved by operating two WDFMs at near maximal speed, which consumed one cup of packed NEFA per generator every 3 hours, or four packed cups per day per chamber, (i.e,. a four-fold higher NEFA consumption than estimated for the chronic exposures). Total aerosol concentration was measured hourly for 4 hours by aspirating a known volume of air through a Gelman Metrical DM-450 membrane filter which was weighed before and after the exposure. The respirable fraction was measured continually, using a portable battery-powered Casella type 113A gravimetric dust sampler. The instrument was suspended above the center of the animal cage in the exposure chamber.

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Within a 30-day observation period after the exposure none of the animals showed any ill effects or died spontaneously. Seven days after the exposure, two randomly selected hamsters were sacrificed to determine the NEFA lung burden, using flame atomic absorption spectrometry (FAAS). The remaining 15 hamsters were sacrificed after the 30-day observation period. The lungs of five of these animals were collected for FAAS analysis. FAAS analysis of the lungs from the two hamsters sacrificed one week after exposure showed an average of 7 µg nickel per lung, indicating a pulmonary deposition and retention of about 78 µg NEFA. The lungs of the five hamsters sacrificed 30 days after exposure contained an average of 6.8 µg nickel per lung, or 75 µg NEFA. These estimates are based on a 9% w/w nickel content of the NEFA and on the assumption that the nickel in the NEFA is retained and does not leach appreciably from the NEFA in tissue fluids. This assumption is supported by the observation that the average nickel content per lung remained constant from 7 days postexposure (7 µg) to 30 days postexposure (6.8 µg), which would not be the case if the nickel were to leach from the NEFA.

The lungs of the remaining 10 animals and of 5 untreated controls were gently perfused through the trachea with 10% neutral buffered formalin to approximately normal volume, followed by tracheal ligation. Routinely processed, paraffin-embedded sections of lung, stained with hematoxylin and eosin, were histopathologically examined. The results are summarized in Table 5. An accumulation of dark brown granular pigment, graded very small or small and resembling NEFA, was present in the lungs of the majority of the NEFA-treated hamsters. This pigment was primarily phagocytosed in macrophages located in areas of bronchiolization of alveolar epithelium. Occasionally the pigment was also present in the interstitial areas. Bronchiolization of the alveolar epithelium was present in both the NEFA-treated hamsters and controls, but appeared to have been increased in the NEFA-treated hamsters. No other treatment-related changes were observed.

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TABLE 5. SUMMARY OF HISTOLOGIC LUNG CHANGES IN HAMSTERS AFTER ONE 6-hr EXPOSURE TO NEFA AEROSOL*

Exposure Group and Animal Number	Histology Number	Histologic Changes in the Lungs**	
		Bronchiolization	Pigment
Control #1	1356-277	0	0
2	1357-277	0	0
3	1358-277	1	0
4	1359-277	0	0
5	1360-277	1	0
NEFA #2	1361-277	1	1
4	1362-277	1	1
6	1363-277	0	0
7	1364-277	1	1
8	1365-277	0	0
11	1366-277	2	2
13	1367-277	1	1
14	1368-277	2	2
15	1369-277	1	1
16	1370-277	1	1

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* Mean respirable aerosol concentration ~220 μg/ℓ

** Key:
0 = Normal
1 = Very slight (or very small amount)
2 = Slight (or small amount)
3 = Moderate
4 = Marked
5 = Extreme

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SUBCHRONIC TOXICITY TEST

MATERIALS, METHODS, AND EXPERIMENTAL PROCEDURES

The hamsters tolerated the 6-hour exposure to 220 μg/ℓ respirable NEFA during the acute toxicity test well. No severe lung changes were observed. It therefore became necessary to determine their tolerance to longer exposures (2-month subchronic toxicity test) at the same high NEFA aerosol concentration in order to determine suitable aerosol concentrations for the chronic exposures. However, generating such a high aerosol concentration (total aerosol concentration ~590 μg/ℓ) consumed NEFA at a rate four times higher than anticipated. This presented the following complications: 1) The one exposure technician budgeted for the project would not have been able to pack the required 8 WDFM cups per day and perform the necessary daily aerosol monitoring. 2) Our NEFA supply would have been consumed during the first few months of the chronic exposure phase unless additional quantities of NEFA were produced at considerable additional expenses. To solve this problem and to increase the respirable fraction of the aerosol after it had been generated by the WDFM, a small fluid energy mill (Trost Gem T Research Model Jet Mill, Garlock, Inc., Plastomer Products Div., Trost Air Mill Dept., Newtown, PA) was placed between the WDFM and the rest of the system (Figure 5). The Trost mill takes dust fed by gravity or airstream and entrains it into two opposing, turbulent jets. The particles collide with one another until small enough to escape the impaction chamber. The result was an increase of at least 50 μg/ℓ in the respirable fraction of the aerosol generated by the WDFM. This system had to be calibrated and adjusted as the 2-month subchronic exposure began. A cyclone elutriator, positioned between the Trost mill and the exposure chamber, removed most of the particles with aerodynamic diameters larger than 10 μm. While the Trost mills significantly increased the efficiency of the aerosol generation and thus served their intended purpose, we did encounter some difficulties in the beginning in maintaining a reasonably stable and reproducible aerosol concentration at the desired levels.

Sixty 6.5-month-old hamsters were randomly divided into 3 groups of 20 hamsters each. Group 1 was exposed for 6 hours/day, 5 days/week for 2 months to a respirable NEFA aerosol concentration intended to be approximately 200 μg/ℓ. Group 2, serving as control, was treated identically but was exposed to FA aerosol rather than NEFA aerosol. Group 3, otherwise treated identically, served as an untreated control group. The exposures started on October 10, 1977, as soon as reasonably stable aerosol concentrations were achieved, and ended on December 9, 1977.

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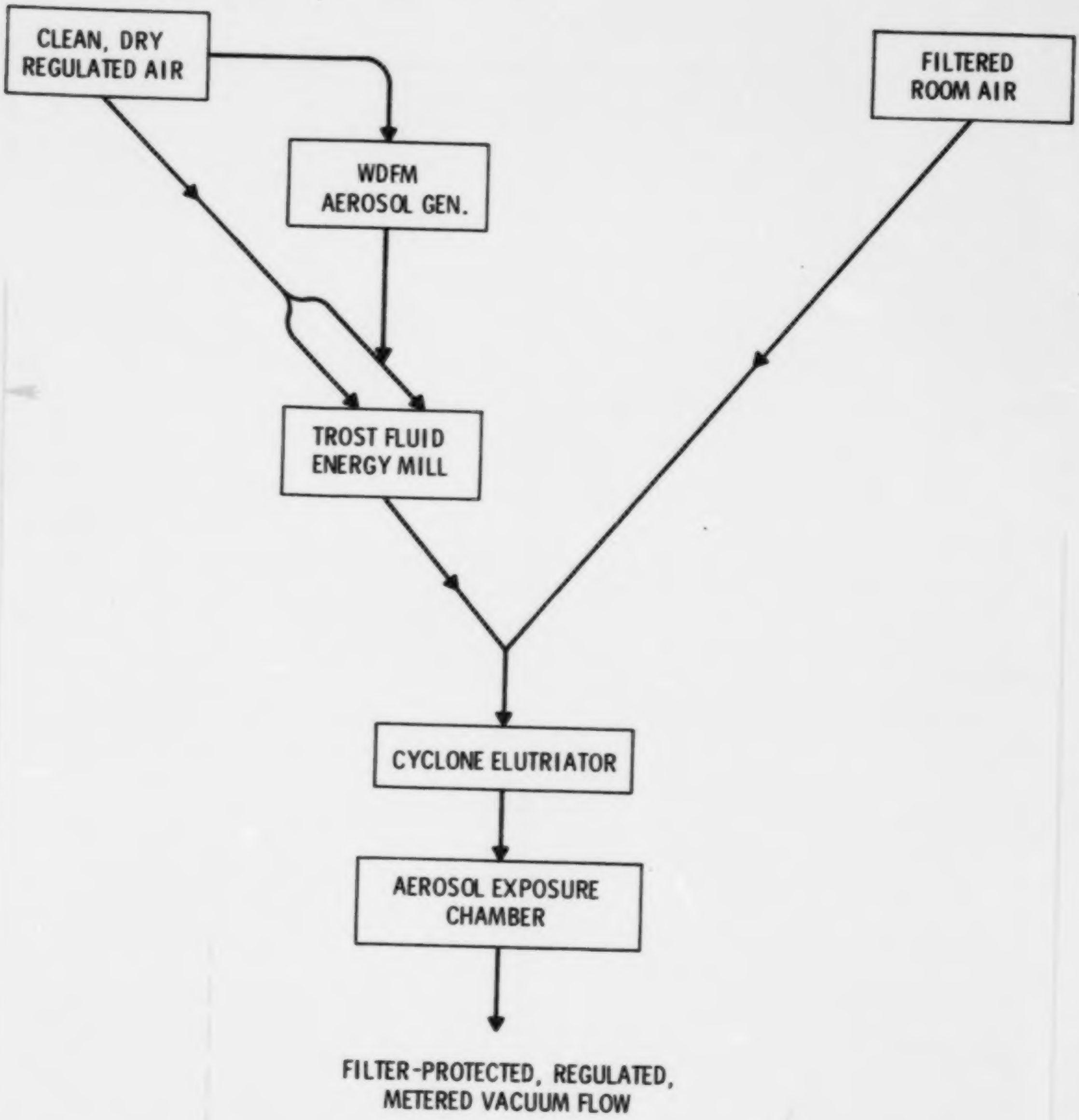

FIGURE 5. Schematic diagram of aerosol exposure system.

The dust preparation prior to packing of the dust into the cups of the WDFM consisted of sieving, using a 212 µm sieve, and heated (100°C) vacuum desiccation. The key variable in the fly ash preparation was the moisture content. Any increase resulted in a decrease in the respirable aerosol fraction due apparently to increased coagulation of the particles.

Total aerosol concentration was measured as in the acute toxicity test, namely by aspirating a known volume of air through each of two Gelman Metrical DM-450 membrane filters which were weighed before and after the measurements. The filters were positioned opposite one another, approximately 45 cm centripetally from the chamber walls, and about 45 cm above the cages. Three 15-minute samples were collected during each 6-hour exposure period, namely 30 minutes after starting the exposures, 2.5 hours later, and before ending the exposures. The respirable fraction was measured simultaneously with the total aerosol concentration measurements, but for 20 minutes each, using a portable battery-powered Casella type 113A gravimetric dust sampler. This device samples at a flow rate of 2.5 ℓ/min and selectively transmits particles which conform to the British Medical Research Council's definition of a "respirable" aerosol. The instrument was suspended above the center of the animal cage in the exposure chamber. Particle size distribution was determined using an Andersen cascade impactor (Andersen, 1966).

The mean weekly total and respirable aerosol concentrations for FA and NEFA are shown in Figures 6 and 7, respectively. The mean respirable FA aerosol concentration in the FA exposure chamber during the 40 exposure days was 183 ± 27 µg/ℓ (SD) compared to 188 ± 49 µg/ℓ in the NEFA chamber. During the first weeks of exposure the air flow rate through the chambers was equal to 4 air changes/hour. In the course of regulating aerosol concentrations at the desired level, this was subsequently reduced to 3 air changes/hour. Since each 2700-ℓ exposure chamber (capacity = 102 hamsters) was occupied by only 20 hamsters, 3 air changes/hour were considered sufficient to maintain satisfactory environmental conditions for the animals. Both the NEFA and the FA aerosol particles consisted of spheres and small flakes, presumably carbon (Figures 8, 9).

Starting 64 hours after completion of the 2-month exposures, all surviving animals were sacrificed by intraperitoneal injection of sodium pentothal and exsanguination. Lungs with the trachea were then removed and the carcass discarded. The lungs of half the animals from each group were gently perfused through the trachea with 10% neutral buffered formalin to their approximate original volume, followed by tracheal ligation. After fixation, the lungs were embedded in paraffin, stained with hematoxylin and eosin, and sectioned for histopathologic examination. The remaining lungs were analyzed for Ni to determine the quantity of NEFA retained in the lungs, using FAAS.

RESULTS AND DISCUSSION

The group mean body weights (MBW) as a function of time and treatment for the NEFA, the FA, and the control animals are shown in Figure 10. The

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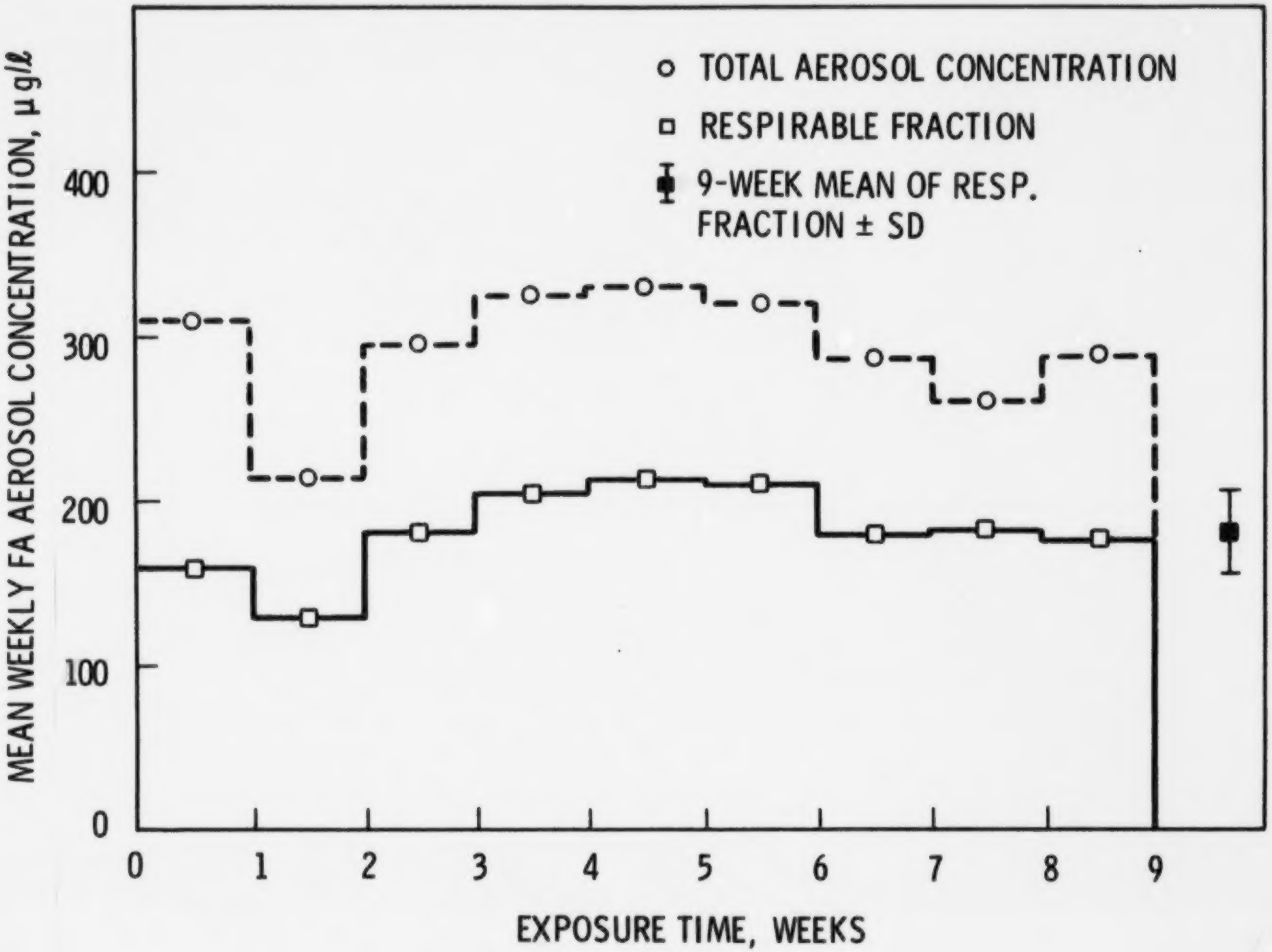

FIGURE 6. Mean weekly FA aerosol concentrations.

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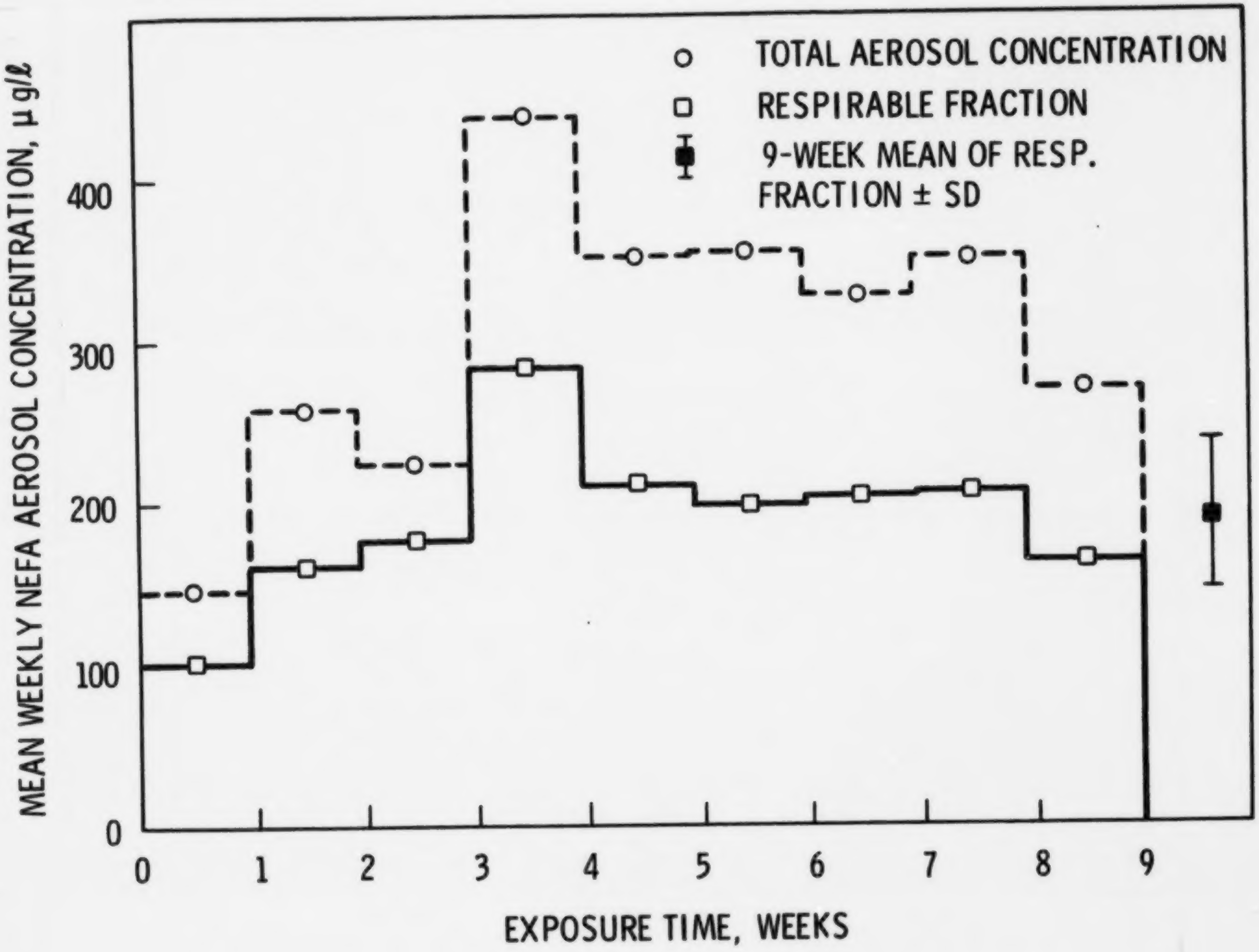

FIGURE 7. Mean weekly NEFA aerosol concentrations.

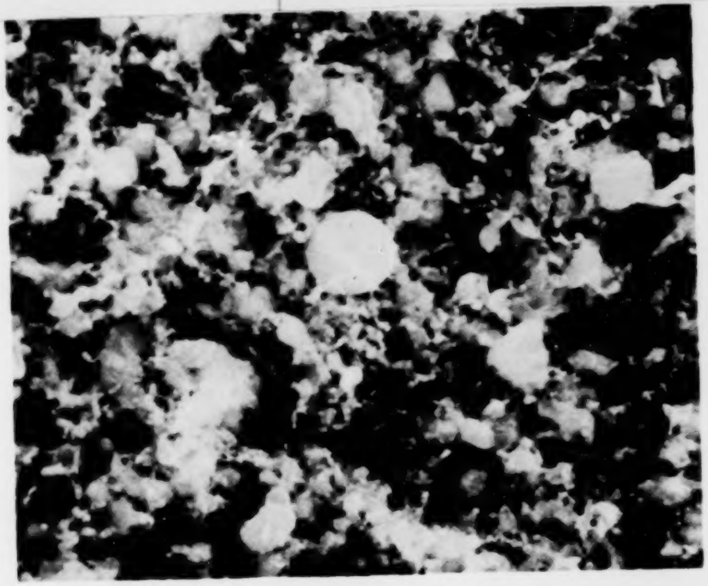

FIGURE 8. Scanning electron micrograph of fly ash particles, 3000 X.

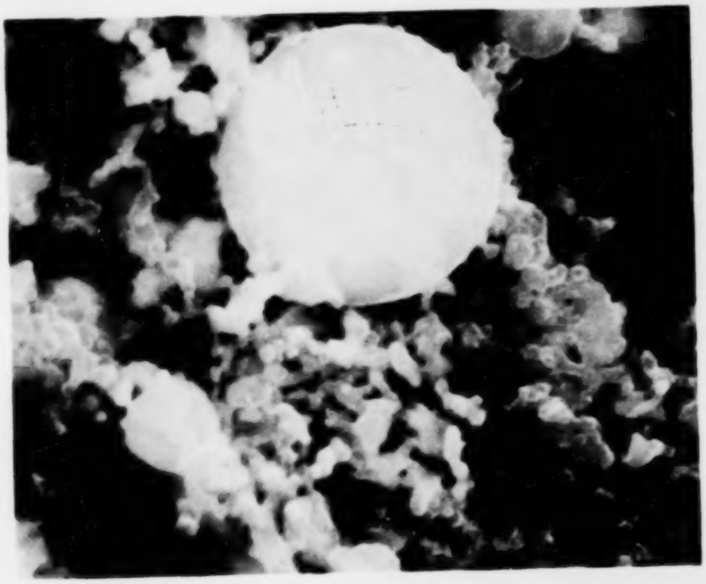

FIGURE 9. Scanning electron micrograph of fly ash particles, 10,000 X.

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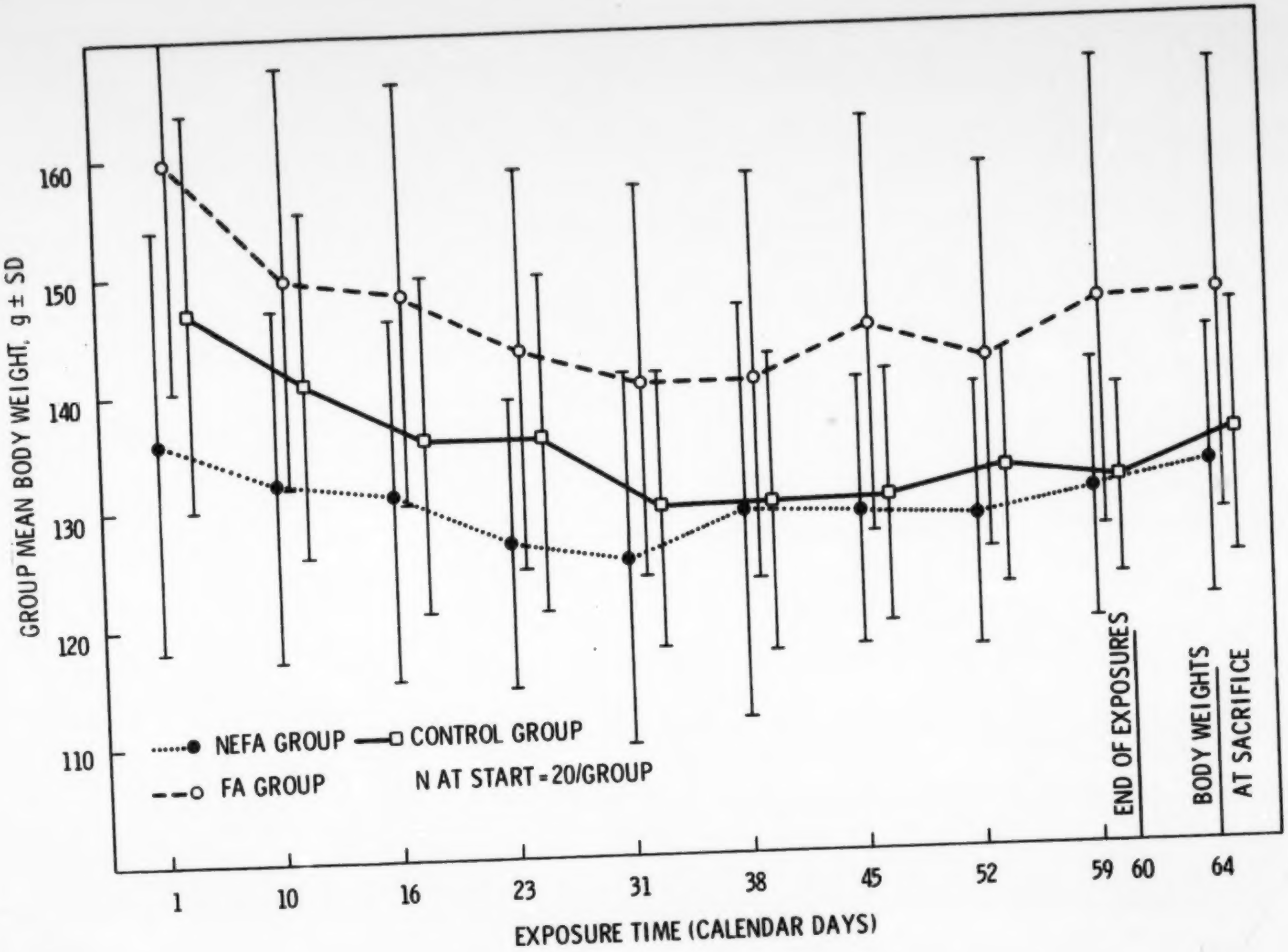

FIGURE 10. Group mean body weights of male Syrian Golden hamsters as a function of time and treatment.

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pre-exposure MBW of the FA group was higher, and that of the NEFA group was lower, than that of the controls. This difference was maintained to varying degrees throughout the 2-month exposure. A repeated-measures analysis of the weight data using a least significant difference multiple comparison procedure shows that the FA animals were significantly heavier ($P < 0.05$) than the other groups. Since this group was significantly ($P < 0.05$) heavier than the others at the first (pre-exposure) measurement and since the curves of MBW as a function of time are fairly parallel, it can be concluded that these differences are not treatment-related. To avoid initial differences in MBWs among the groups of the chronic exposure experiment, the animals were assigned to these groups by stratified random selections.

All three weight curves showed a gradual initial decrease, followed by a relatively weight-stable period. The dust exposures can be ruled out as a causative factor for the initial decrease in MBWs as the controls showed the same behavior. Perhaps the stress of transferring the animals from plastic group cages to the individual wire mesh cages 3 days before the start of the exposures might account for this phenomenon.

During the 2-month exposure period four control animals and one NEFA-exposed animal died of undetermined but apparently treatment-unrelated causes. The NEFA-exposed animal had developed ascites. Its girth increased considerably and its death weight was 188 g. At necropsy, 7 to 8 ml of fluid were found in the abdominal cavity. The surviving animals showed no apparent ill effects.

At necropsy after 2 months of exposure, the lungs of the FA- and NEFA-exposed hamsters were black or dark brown in contrast to the normal pink color of the lungs from the control hamsters (Figure 11). Results of the histopathologic examination are summarized in Table 6. Black or dark brown granular dust was scattered in alveoli throughout the lung sections but appeared somewhat more concentrated in subpleural and peribronchiolar areas (Figure 12). The dust was generally phagocytosed by alveolar macrophages with the macrophages so heavily laden with dust that normal cellular details were obscured (Figure 13). Aside from the increased number of macrophages with phagocytosed dust, there was minimal inflammatory reaction. Slight thickening of alveolar septa and some bronchiolization of alveolar epithelium was also apparent. No differences between the FA- and NEFA-exposed groups were observed.

Weights of the wet (fresh) lungs analyzed for nickel and the results of the FAAS analyses are shown in Table 7. One-way analysis of variance of the wet lung weights shows that the means of the three groups are not equal. Scheffe's S method was used to make all possible comparisons among means. Results of these comparisons show that the FA and NEFA mean wet lung weights are not significantly different from one another but are significantly ($P < 0.001$) higher than that of the controls. The higher lung weights of the dust-exposed animals are probably due to the slight degree of interstitial reaction found in these lungs (Table 6). An average of approximately 510 μg nickel per lung was found in the hamsters after 2 months of exposure to NEFA at a mean respirable aerosol concentration of about 190 μg/ℓ. Quantities of nickel in the FA-exposed animals and in the controls were below the detection

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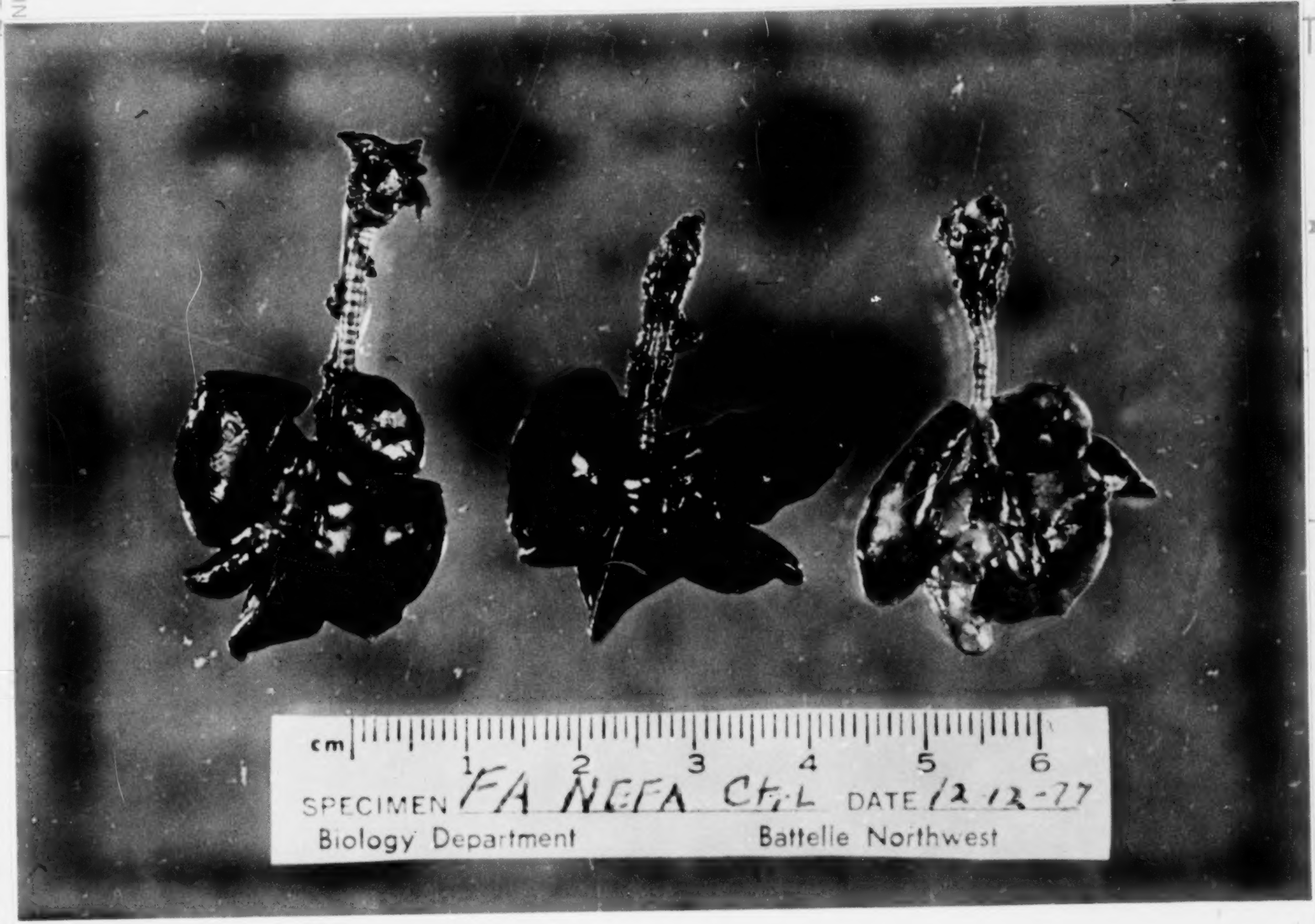

FIGURE 11. Lungs of hamsters after 2-month exposures to FA aerosol (left) and NEFA aerosol (center). Lung of control animal is shown at right.

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TABLE 6. SUMMARY OF HISTOLOGIC LUNG CHANGES AFTER 2 MONTHS OF EXPOSURE TO NEFA AND FA AEROSOLS*

Exposure Group and Animal #	Lung Changes** Dust	Interstitial Reaction	Bronchiolization	Miscellaneous
NEFA				
1	3	2	1	
3	3	2		
5	3	2	1	
7	3	4		Atelectasis (died)
7	3	2		
9	4	2		Very slight emphysema and pleuritis
11	3	2		
12	3	2		
14	3	2		
15	3	2		
16	4	2	1	
FA				
1	3	2		
2	3	2		
3	3	2		
5	3	2		
7	3	2		
8	3	2		
10	3	2		
12	3	2	1	Bronchiolar villi
14	4	3		
15	3	2		
Sham-Exposed Controls				
1		1	1	
3				
5	1			
9				
11			2	
13				Slight Leukocytosis
15				
16			1	
17		1	2	

---

* Mean respirable aerosol concentration ~185 μg/ℓ

** Key: Blank = Normal
1 = Very slight (or very small amount)
2 = Slight (or small amount)
3 = Moderate
4 = Marked
5 = Extreme

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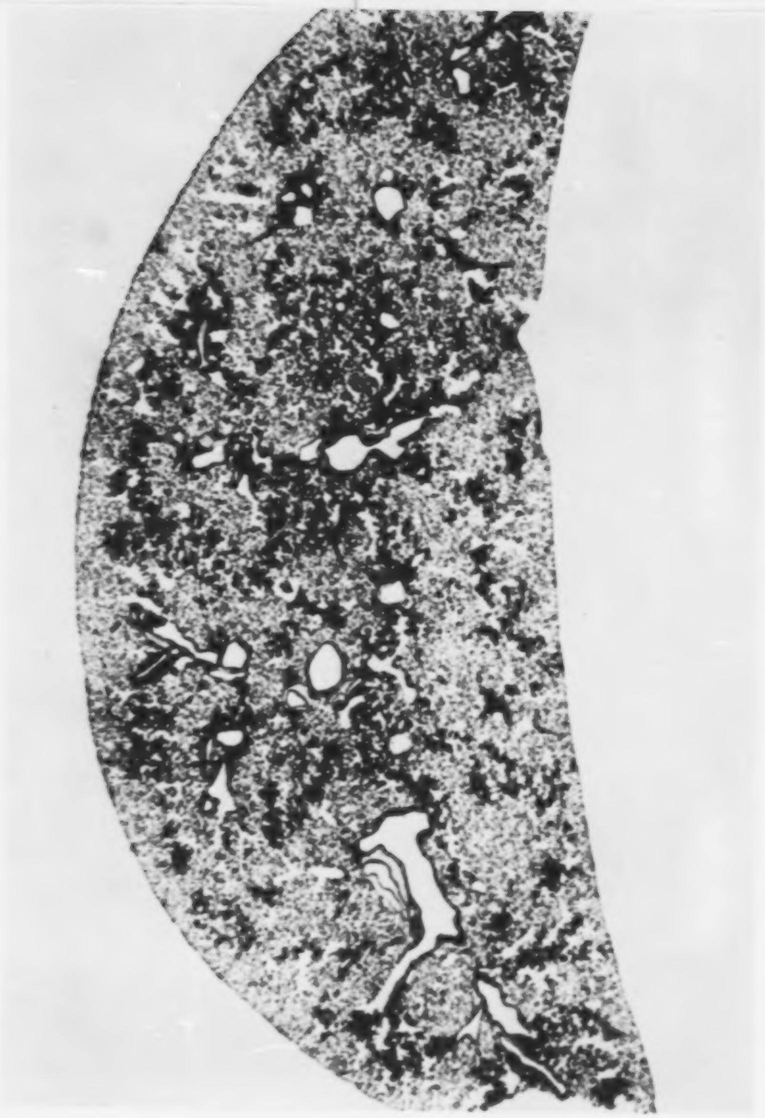

FIGURE 12. Lung section of hamster after 2-month exposure to NEFA aerosol, showing dark granular dust scattered in alveoli throughout the lung with local concentrations in subpleural and peribronchiolar areas. H&E stain, 12.5 X.

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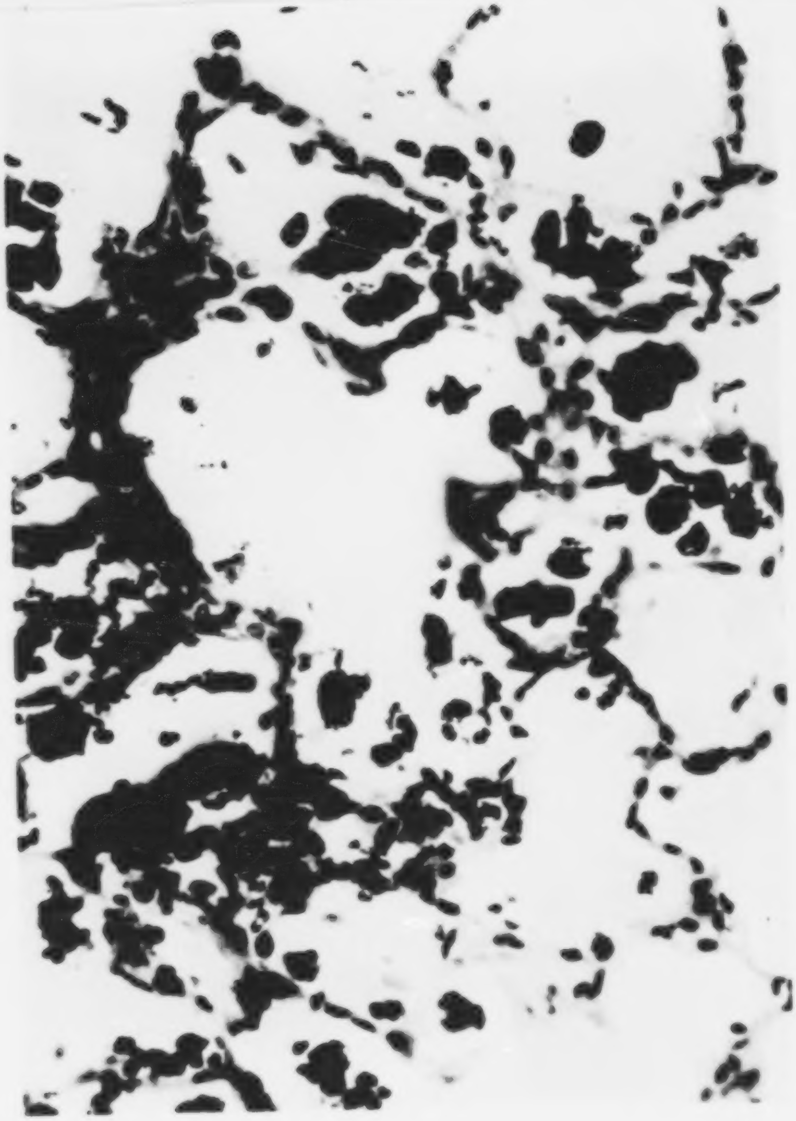

FIGURE 13. Lung section, similar to the one shown in Figure 12, at higher magnification, showing many dust-laden alveolar macrophages. H&E stain, 500 X.

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TABLE 7. BODY WEIGHTS, WET LUNG WEIGHTS AND QUANTITIES OF NICKEL FOUND BY FLAME ATOMIC ABSORPTION SPECTROSCOPY IN THE LUNGS OF HAMSTERS AFTER 2 MONTHS OF EXPOSURE TO NEFA AEROLSOLS.

Animal Identification	Body Weight (g)	Wet Lung Weight (g)	Lung Weight/ Body Weight	Ni in Lung (μg)	Equivalent mg Fly Ash*
Cage 1, Controls					
#2	--	1.120	--	<10†	--
4	132	0.921	0.00698		--
6	147	0.928	0.00631		--
8	133	0.900	0.00677		--
10	123	0.809	0.00658		--
12	142	0.987	0.00695		--
14	132	0.967	0.00733		--
17	119	0.806	0.00677		--
Mean ± SD	133 ± 10	0.930 ± 0.101	0.0068 ± 0.003		
Cage 2, FA					
#1	146	1.370	0.00938	<10†	--
2	126	1.410	0.01137		--
4	166	1.810	0.01090		--
6	106	1.403	0.01324		--
7	149	1.444	0.00969		--
9	134	1.621	0.01210		--
11	138	1.377	0.00998		--
13	126	1.495	0.01187		--
16	130	1.524	0.01172		--
17	154	1.648	0.01070		--
Mean ± SD	138 ± 17	1.510 ± 0.143**	0.0110 ± 0.0012		
Cage 1, NEFA					
#1	147	1.673	0.01138	612	6.80
3	118	1.503	0.01274	502	5.58
5	136	1.293	0.00951	385	4.28
8	149	1.529	0.01026	550	6.11
10	128	1.326	0.01036	467	5.19
12	134	1.386	0.01034	521	5.79
14	112	1.420	0.01268	488	5.42
15	142	1.515	0.01067	475	5.28
16	134	1.547	0.01154	596	6.62
Mean ± SD	133 ± 12	1.466 ± 0.120**	0.0111 ± 0.0111	511 ± 70	5.67 ± 0.77

---

* Calculated assuming 9% w/w nickel in NEFA

** Significantly different from controls, $P < 0.001$

† Limit of detection ~10 μg per processed lung

Note: The mean body weights in Table 7 may vary from those shown in Figure 10 because in Figure 10 the mean body weights of all hamsters/group are presented.

limit of the method, i.e., <1 μg. Based on a 9% w/w nickel content of the NEFA, and assuming that there was no appreciable leaching of nickel from the NEFA, the average quantity of NEFA retained per lung in the NEFA-exposed hamsters after 2 months of exposures (40 six-hour exposures) was approximately 5.7 mg.

Neither the acute nor the subchronic exposures had any noticeable effect on the well-being or body weight of the exposed animals although the respirable fractions of the aerosol concentrations were very high, namely 220 and 190 μg/ℓ, respectively.

Substantial quantities of NEFA were inhaled and retained in the lungs as evidenced by the dark color of the lungs at necropsy (Figure 11), the histopathologic findings (Table 6, Figure 12), and quantitatively by the FAAS analyses (Table 7). None of the trace elements naturally occurring in FA were present at levels sufficiently high to permit FAAS analysis. While no quantitative data were available for the amount of FA present in the lungs of the FA-exposed animals, it is reasonable to assume that the quantities of FA retained in the lungs were very similar to those of NEFA as determined by FAAS. This statement is based on the observations that (a) NEFA and FA particles have very similar particle size characteristics and can thus be assumed to have very similar aerodynamic (and thus pulmonary deposition) characteristics; (b) at necropsy, the lungs of the NEFA- and of the FA-exposed animals looked equally dark; and (c) histopathologically, the grading of dust found in the lungs of the NEFA- and of the FA-exposed animals was very similar (Table 6).

There was no apparent difference between the effects of NEFA and those of FA under the investigated conditions of acute and subchronic exposures.

The pulmonary retention of substantial quantities of NEFA and FA dust notwithstanding, histopathologic examination revealed only a nonspecific dust reaction (mild pneumoconiosis) even after 2 months of exposure. This indicated a relatively low acute and subchronic toxicity of both NEFA and FA in the hamster lung. Similar findings were observed in our laboratory when hamsters were exposed to NiO (Wehner et al., 1972, 1975) 6 hours/day, 5 days/week, for 3 weeks (mean respirable NiO aerosol concentration ~40 μg/ℓ); for 3 months (~60 μg/ℓ); and for life (53 μg/ℓ). None of those animals died during the 3-week and the 3-month exposures. Of the animals receiving lifelong exposures, those that died during the first few months (from causes unrelated to the exposures) had heavy burdens of NiO but showed relatively little cellular response. Both proliferative and inflammatory cellular response increased only later in the lifelong exposure group, leading eventually to severe pneumoconiosis with severe degrees of lung consolidation. We found that NiO had low solubility in tissue fluids and relatively long residence time in the lung; 45 days after exposure, 45% of the NiO deposited in the lung was still present. By contrast, exposure of hamsters to CoO (mean respirable aerosol concentration ~110 μg/ℓ) 3 hours/day resulted in the death of all hamsters within 4 days (Wehner et al., 1972). Within 7 days, essentially all the CoO had been cleared from the lungs of other hamsters exposed to CoO, while more than 70% of the NiO was still present at that time.

Based on a 9% w/w nickel content of the NEFA, and assuming that there was no appreciable leaching of nickel from the NEFA, the average quantity of NEFA retained in the lungs of the NEFA-exposed hamsters after 2 months of exposures (40 six-hour exposures) was approximately 5.7 mg. By comparison, one 6-hour exposure (acute toxicity test) showed an average of 7 μg nickel per lung, or 78 μg NEFA, in the two hamsters sacrificed one week after exposure, and 6.8 μg nickel per lung, or 75 μg NEFA, in the five hamsters sacrificed 30 days after exposure. Thus, the 40 exposures of the subchronic toxicity test resulted in a 76-fold higher pulmonary NEFA retention than the one exposure of the acute toxicity test, i.e., almost twice as much nickel was found in the lungs of the subchronic exposure animals as might be expected based on the acute toxicity test, not considering any clearance mechanisms. The following explanations might be considered to account for this difference between observed and expected lung burden.

(1) The time from completion of the exposures to sacrifice (64 to 70 hours) was not long enough to allow for mucociliary clearance of nickel deposited in the ciliated part of the respiratory tract. The observed pulmonary NEFA burden of 5.7 mg would thus comprise the deep lung burden plus some quantity not yet cleared by mucociliary action. However, our experience in the past indicates that mucociliary clearance in hamsters is usually complete within a period shorter than 64 to 70 hours, given a normally functioning clearance mechanism.

(2) Repeated 6-hour exposures to such high aerosol concentrations might have "overwhelmed" the clearance mechanism, making it less and less efficient, i.e., resulting in larger quantities of material retained.

(3) The difference of a factor of ~2 between observed and expected lung burdens falls within the inherent variations of such biological systems.

(4) The hamsters of the acute toxicity test were 4 months old; those of the subchronic toxicity test were almost 7 months old. However, their average body weights were similar, namely 126 g for the former and from 133 to 147 g for the latter groups. This indicates that the 4-month-old group had reached adulthood and thus can be assumed to have respiratory function and pulmonary deposition/retention characteristics similar to those of the 7-month-old group.

The percentage of inhaled NEFA that was deposited in the deep lung may be estimated from the following known parameters: respirable aerosol concentration, exposure time, minute volume of the hamster (60 ml; Gleysteen and Stroud, 1974), and the quantity of NEFA deposited in the lungs of the exposed animals. These calculations indicate that during the 6 hours of exposure to 220 μg/ℓ respirable NEFA aerosol the animals inhaled an average of approximately 4.75 mg NEFA of which approximately 80 μg, or less than 2% of the inhaled quantity, was deposited in the deep lung.

As already mentioned, the mean wet lung weights of both the FA- and the NEFA-exposed groups were significantly ($P < 0.001$) higher than that of the controls, namely, 1.51 and 1.47 g versus 0.93 g, respectively (Table 7). If the assumption is correct that the lung weight increase is due to early lung changes (interstitial reaction) as a consequence of the NEFA and FA exposures (Table 6) lung weights might be a more sensitive criterion for early changes than the histopathologic changes observed under the microscope. The results of the chronic exposures (q.v.) support the findings reported here.

In conclusion, it can be stated that the results of the acute and subchronic exposures indicate relatively low acute and subchronic toxicity of NEFA and FA in the hamster with the qualification that the mild pneumoconiosis, caused by the dust exposures, resulted in a highly significant increase in wet lung weights for the exposed animals. Our findings suggest relatively low solubility of the substances in body fluids. The latter assumption was subsequently confirmed by a pulmonary deposition, translocation and clearance study with neutron-activated fly ash (q.v.). Enrichment of fly ash with nickel did not enhance its biological effects in hamsters under the described conditions, but facilitated convenient determination of lung burdens. Wet lung weights might be a sensitive criterion for early lung changes.

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THE CHRONIC EXPOSURE EXPERIMENT

MATERIALS, METHODS AND EXPERIMENTAL PROCEDURES

After a 3-week quarantine period, 408 2-month-old hamsters were randomly divided into 4 groups of 102 hamsters each. To avoid initial mean body weight differences among the experimental groups, the animals were assigned to their groups by stratified random selection. Group 1 was exposed for 6 hours/day, 5 days/week to a respirable aerosol concentration of 70 μg/ℓ NEFA. Group 2 was exposed to NEFA aerosol under conditions identical to those for Group 1, except for a lower respirable aerosol concentration, namely 17 μg/ℓ. Group 3, serving as FA control and otherwise treated identically, was exposed to 70 μg/ℓ FA aerosol. Group 4, otherwise treated identically, served as the sham-exposed control group. Sham exposures consisted of exposing the animals to filtered room air at a flow rate similar to those in the NEFA and FA exposure chambers, but the air did not contain NEFA or FA. The aerosol concentrations were selected in consultation with Dr. Moore, then EPA Project Officer, based on the results of the subchronic toxicity experiment. The exposures started on January 16, 1978.

From each of the four groups of 102 hamsters, randomly selected groups of 5 hamsters were sacrificed after 4, 8, 12 and 16 months of exposure. All surviving animals were sacrificed after 20 months of exposure. The limited objective of the serial sacrifices was to provide an approximation of lesion developments as a function of dose level and cumulative exposure time. Five animals per serial sacrifice are not intended to provide statistically significant differentiation of results from one serial sacrifice to another in case of only small and gradual histologic changes; however, they are generally sufficient to provide indications of trends, types and degrees of lesion developments.

In similar fashion, randomly selected groups of 5 hamsters were withdrawn from exposure at the same time intervals (after 4, 8, 12 and 16 months of exposure) and maintained for observation for the remainder of their life span up to the 20-month exposure point of their exposed cohorts. The observation period is referred to in this report as "recuperation time" and the animals are referred to as "retired" hamsters. The objective of the serial withdrawals was to obtain an approximation as to whether and to what degree reversal of exposure-induced lesions might occur as a function of dose level, cumulative exposure and recuperation time.

The animals were maintained in the individual 150-cm^2 compartments of stainless steel wire mesh cages (Figure 3) and had free access to Wayne Lab-Blox F4 laboratory animal feed (Allied Mills, Inc., Chicago, IL) and

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water. They were weighed biweekly during their growth period and monthly thereafter. The cages of the exposed and sham-exposed animals remained in the 2700-ℓ exposure chambers (Figure 4) for the duration of the experiment except for brief periods of weekly cage changing, and weighing of the animals. To randomize any biased environmental conditions that might have existed between different positions of the cages within each exposure chamber, the cages were rotated from exposure to exposure. Those animals that were withdrawn from exposure were maintained in the described cages, but the cages were placed on standard cage racks in the exposure rooms. All animals were checked before and after each exposure.

A complete necropsy was performed on each hamster found dead or sacrificed. Moribund animals were sacrificed. Sacrificed animals were killed with an intraperitoneal injection of sodium pentothal. Body weights and (from serially sacrificed animals) lung weights were recorded. The lungs were fixed in gluteraldehyde at 25 cm of hydrostatic pressure and volumes measured, using the water displacement technique. Additional tissues were fixed in 10% formalin. These tissues included liver, kidney, heart, larynx, trachea, thyroid, mediastinal tissue with lymph nodes, eyes, adrenal glands, testes, prostate, spleen, pancreas, urinary bladder, intestines, and femur. Routinely processed, paraffin-embedded, H&E-stained sections of each lobe of lung, trachea, mediastinal tissue (usually including lymph node), liver, kidney, and grossly abnormal organs from each hamster were histopathologically examined. In addition, all organs listed above were histopathologically examined in 5 hamsters from each of the 4 treatment groups at the final sacrifice time after 20 months of exposure. Five animals of the total of 408 animals (1 from the high NEFA, 1 from the FA, and 3 from the sham exposure group) were not histopathologically examined due to autolysis.

The lesions were coded using a modified Systematized Nomenclature of Pathology (SNOP) coding system (College of American Pathology, 1969). The severity of each nonneoplastic lesion was graded as follows: 1 = very slight or very small amount, 2 = slight or small amount, 3 = moderate, 4 = marked, and 5 = extreme. The animal identification, exposure parameters, and coded lesions were entered on a CRT* office terminal into a DATATRIEVE system on a PDP 11/70 computer. Statistical evaluation using the Mantel-Haenszel χ^2 method (Mantel and Haenszel, 1959) were performed on selected lesions of the lung, liver, kidney, and mediastinal lymph nodes.

Weighed fractions of lung tissue were collected after the final sacrifice and analyzed for nickel, using FAAS, to estimate total pulmonary nickel burdens.

Criteria for evaluating the effects of the exposures were body weights, mortality, gross and histopathologic findings. Figure 14 shows a summary of the experimental design.

The flow rate of HEPA-filtered room air (mixed with the respective aerosols for the exposed groups) during exposure was approximately 170 ℓ/min, which provided 3.7 air changes per hour in the 2700-ℓ exposure chambers.

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* Cathode ray tube

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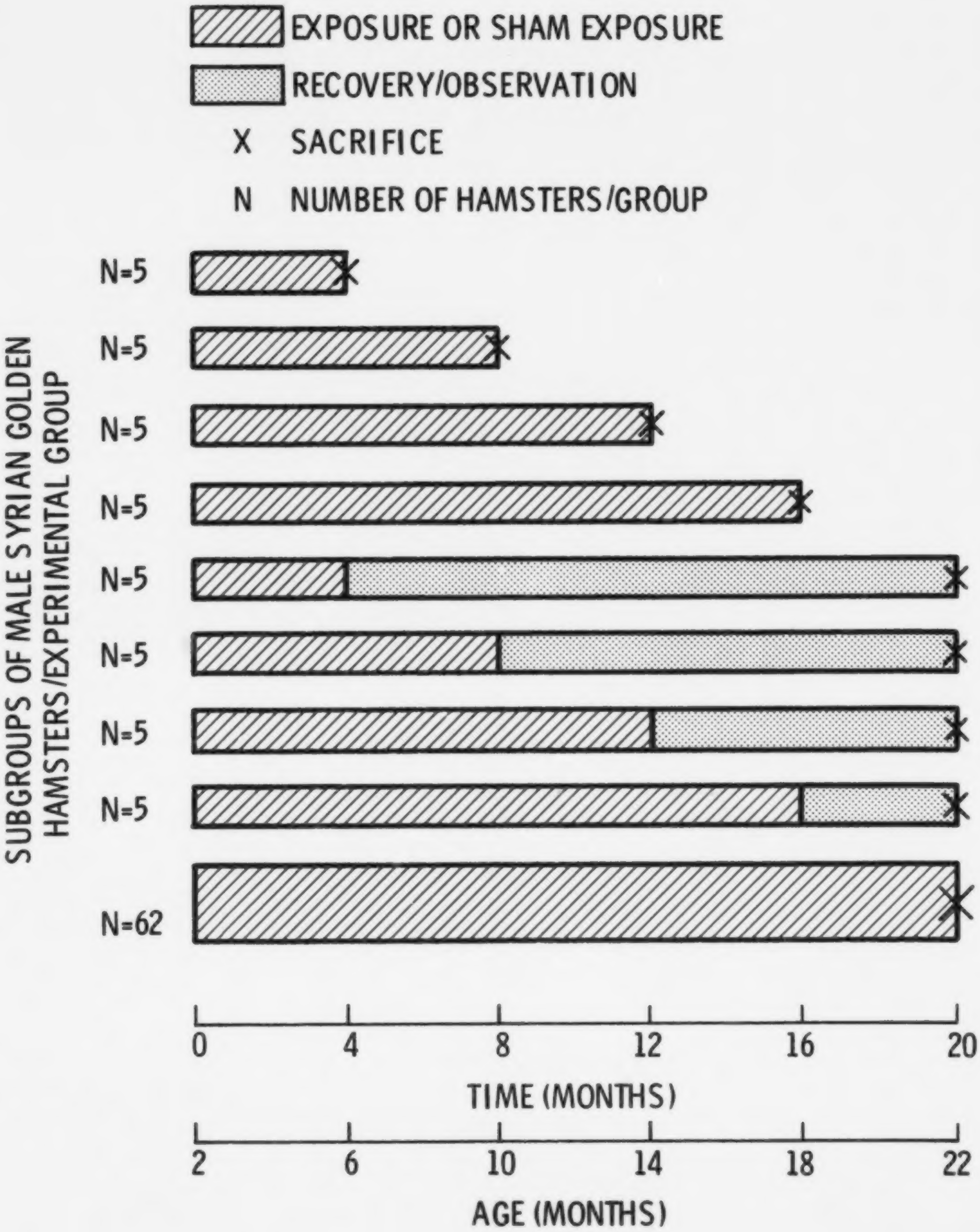

FIGURE 14. Experimental design for each hamster group of the chronic exposure experiment.

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After each daily exposure, each chamber was purged with filtered room air for approximately 30 minutes before the chamber door was opened. The door remained open until the next exposure on the following day. The bottoms of the chambers were washed out daily with the animals remaining in the chamber. Once a week, the entire exposure chamber was cleaned thoroughly while the animals were temporarily removed from the chamber and placed in clean cages. A 300 ppm quaternary ammonia solution, injected into 45°C water, was used for washing and cleaning.

The two NEFA exposure chambers were located in one room of our temperature-controlled animal facilities in the basement of our Life Sciences Laboratory II building which was completed in May 1975. The two chambers for the FA and sham exposures were located in an adjacent identical room.

Temperature and relative humidity were measured as described on page 13. The mean values for each quarter were reported in our quarterly progress reports. For this report, the quarterly means were analyzed using a two-way analysis of variance with the factors being treatment and time. Because the number of observations in each quarter was not constant, the means were weighted by the square root of the number of observations in each mean. However, an unweighted analysis yielded the same results.

The temperature in the sham control chamber averaged about one degree higher than the mean of all four chambers, and the temperature in all chambers was higher than the mean in the second quarter and lower in the fifth quarter of the exposures (Figure 15).

There were no significant differences in relative humidity among the four chambers, but the relative humidity in all chambers tended to be high in the second quarter and low in the fourth quarter of the exposures, reflecting seasonal changes (Figure 16).

The dust preparation, aerosol generation and characterization followed the procedures described for the subchronic toxicity test. In the high-level NEFA and in the FA chamber, two 40-minute samples were collected by each of two probes during each 6-hour exposure period to determine total aerosol concentrations. The samples were collected approximately 60 and 240 minutes after starting the exposures. In the low-level NEFA chamber, one 90-minute sample per exposure was collected.

The respirable fraction of the aerosol was measured simultaneously with the total aerosol concentration, but for 25 minutes each in both the high-level NEFA and the FA chamber, using a portable battery-operated Casella type 113A gravimetric dust sampler. In the low-level NEFA chamber, a 200-minute sample (500 ℓ) was collected daily.

The mass concentration data were analyzed by grouping the weekly means by month. The weekly means were based on approximately the same number of samples, so the variance was constant. The monthly means were then compared using a one-way analysis of variance. This method of analysis will not

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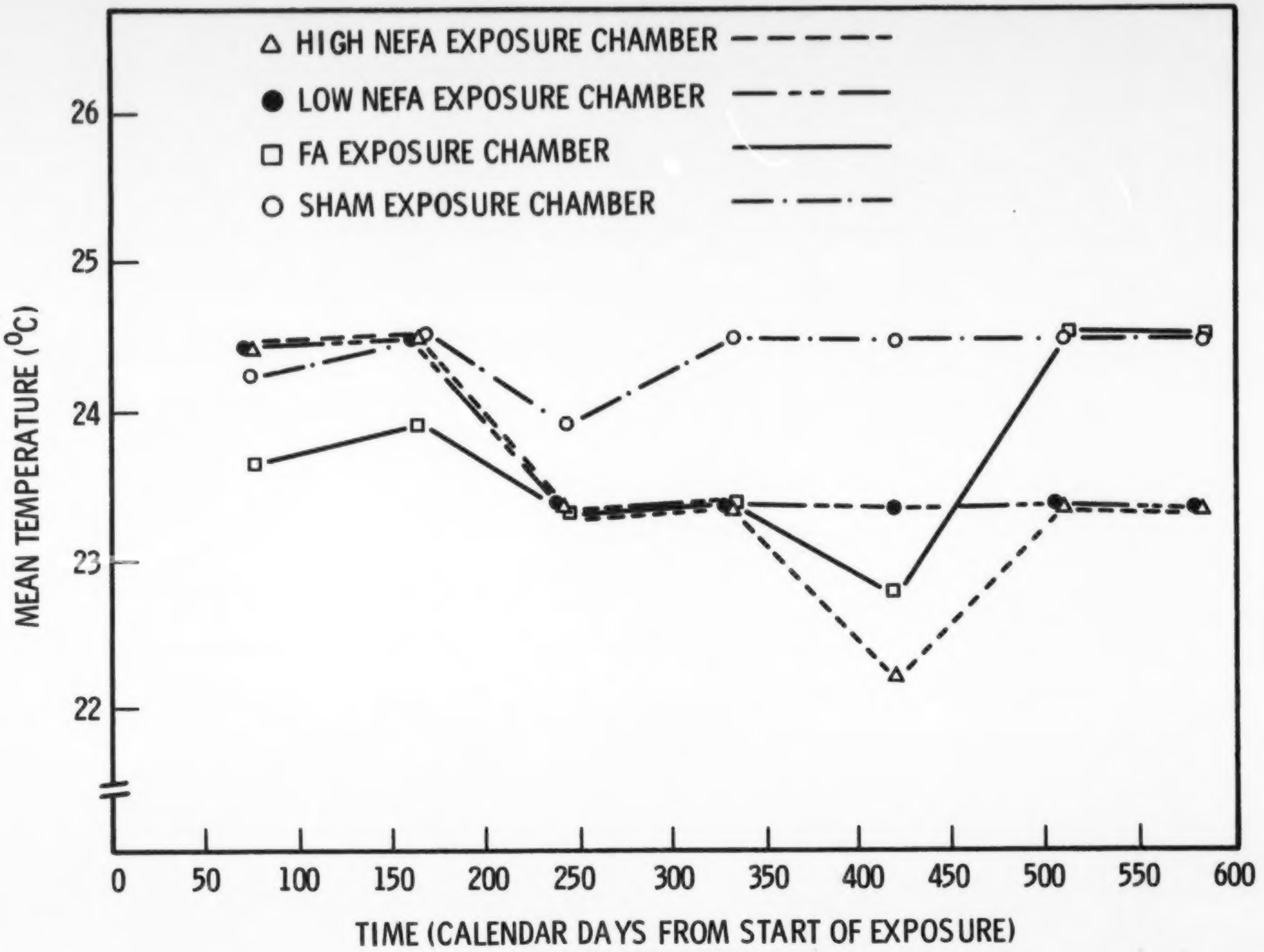

FIGURE 15. Quarterly mean temperatures in the exposure chambers.

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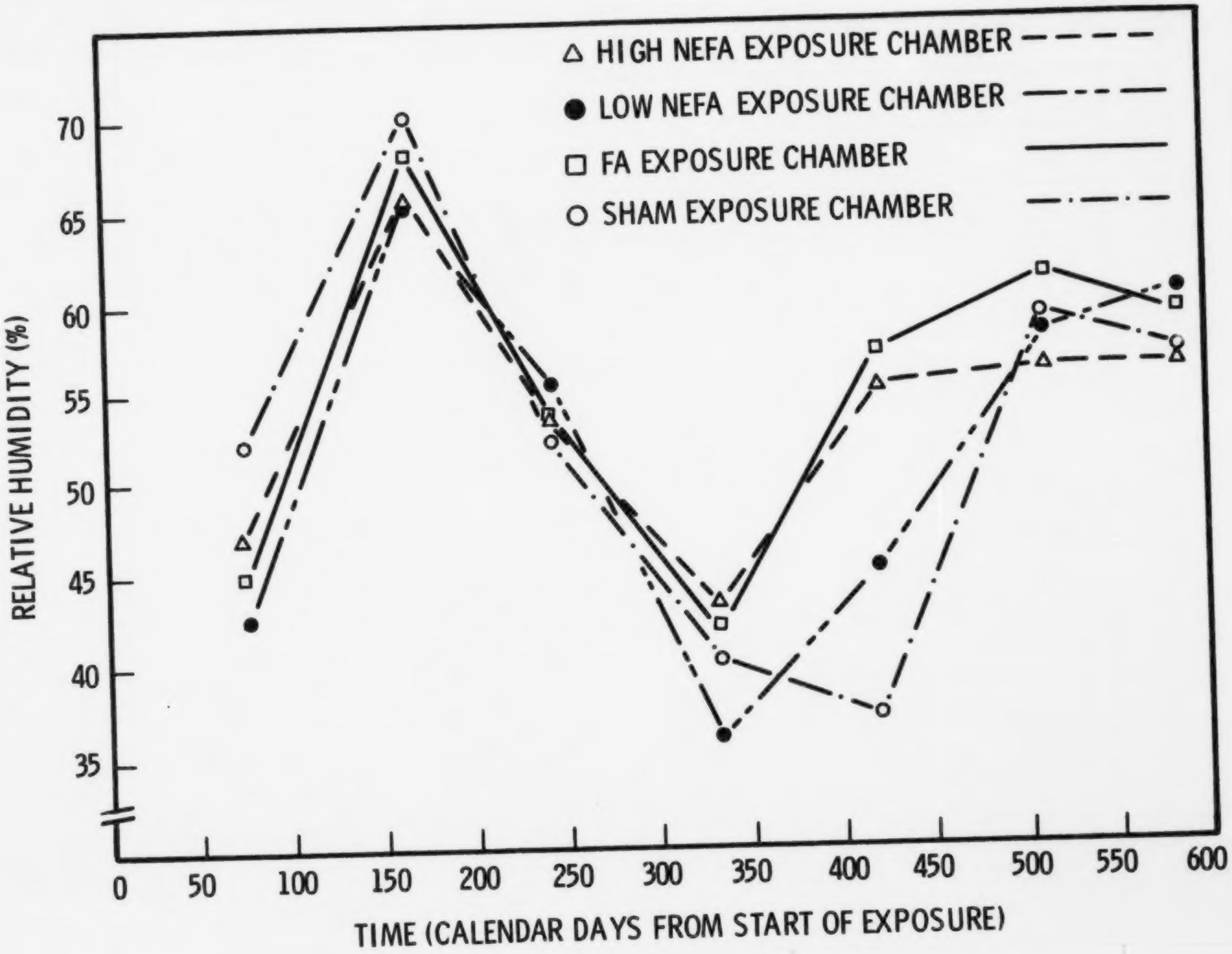

FIGURE 16. Quarterly mean relative humidity in the exposure chambers.

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detect short-term excursions from the mean; however, trends that continued for several weeks should be detected.

All three chamber concentrations showed some evidence of trend in that the "between-months" variation was significantly greater than should arise from the "among-months" variation. Figures 17 through 19 show total and respirable aerosol concentrations as a function of time for the three exposure chambers. Means of the respirable aerosol concentrations for the entire 20-month exposure period were 70.5, 16.6, and 70.5 μg/ℓ for the high NEFA, the low NEFA and the FA chamber, respectively (Table 8).

Cascade impactor samples of the aerosols were collected weekly to determine particle size distributions, using an Andersen (1966) 2000 Inc. Model 20-000 cascade impactor with 8 jet stages and a final filter. Petroleum jelly-coated aluminum foil discs were used as impaction plates to avoid particle bounce. These coated discs were weight-stable after 2 days of curing at room temperature. The impactor was operated at a flow rate of 20 ℓ/min. Sampling time in the high-level (75 μg/ℓ) chambers was 20 minutes, in the low-level chamber 90 minutes. The means (± SD) of the mass median aerodynamic diameters for the 20-month exposure period were 2.8 ± 1.7, 2.8 ± 0.5, and 2.7 ± 0.6 μm for the high NEFA, the low NEFA and the FA aerosols, respectively. The means (± SD) of the geometric standard deviations for the three aerosols were 2.4 ± 0.3, 2.4 ± 0.5, and 2.4 ± 0.3.

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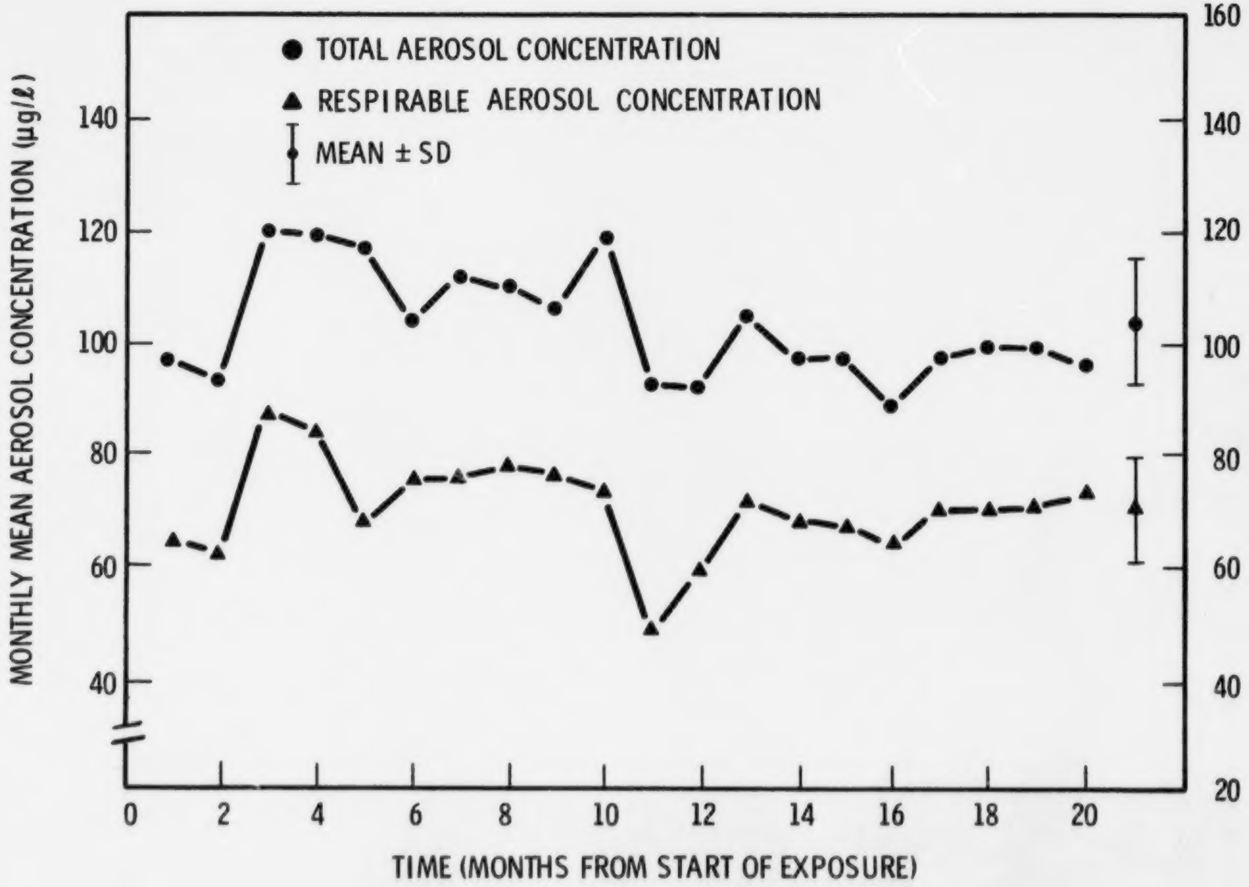

FIGURE 17. Monthly mean aerosol concentrations in the high NEFA exposure chamber.

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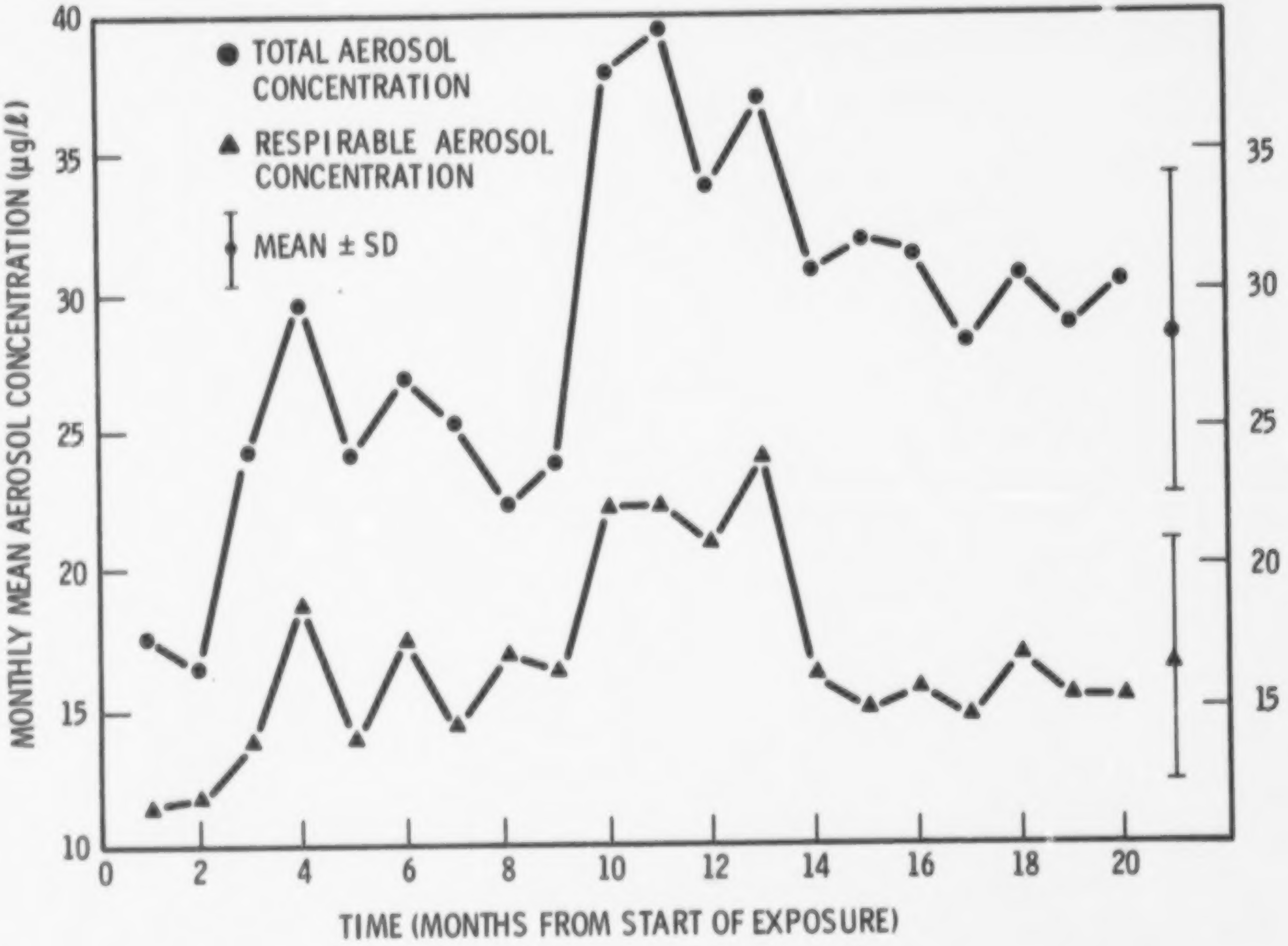

FIGURE 18. Monthly mean aerosol concentrations in the low NEFA exposure chamber.

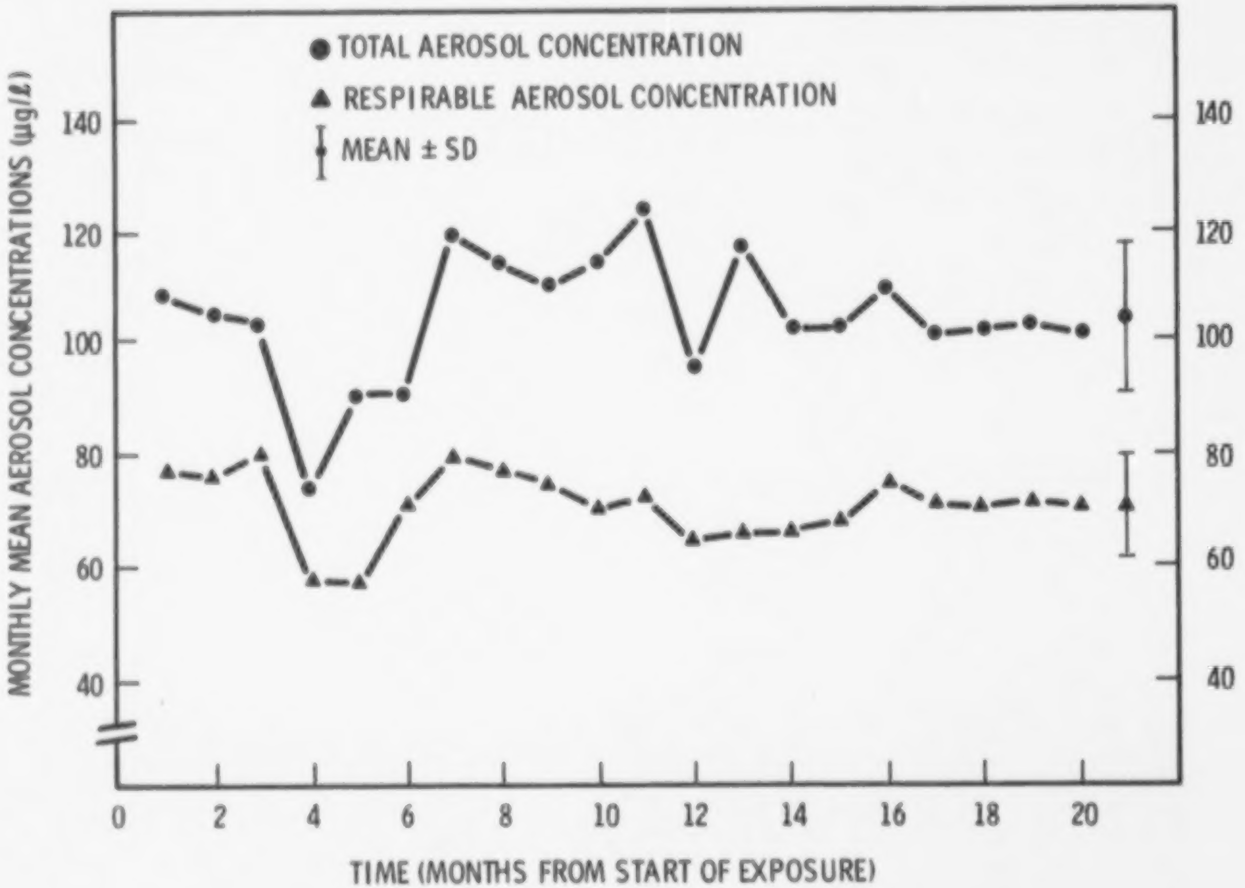

FIGURE 19. Monthly mean aerosol concentrations in the FA exposure chamber.

TABLE 8. SUMMARY OF AEROSOL CONCENTRATION DATA

	Mean ± SD (µg/ℓ)
High NEFA	
Total	103 ± 12
Respirable	70 ± 9
Low NEFA	
Total	28 ± 6
Respirable	17 ± 4
FA	
Total	104 ± 14
Respirable	70 ± 10

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RESULTS

Body Weights

Figures 20 through 23 show the mean body weights (± SD) as a function of time and treatment for the four experimental groups. For convenient comparison, the mean body weights of all four groups are presented together in Figure 24; the bars showing the standard deviations were deleted in Figure 24 to preserve the clarity of the illustration.

Analysis of the body weight data was carried out using the group MBW. Because the size of the group decreased as a function of time, the means were weighted with the square root of the group size in an effort to correct for heteroscedasticity. A two-way analysis of variance model was used to test for treatment effects and time-dependent effects. There were no significant differences among the MBWs ($F = 0.31$, $P > 0.50$). However, as expected, MBWs did change significantly as a function of time. As they matured, the hamsters gained weight from the beginning of the exposure until 400 to 500 days of exposure. Subsequently, the group mean weights declined somewhat as the hamsters aged.

This general pattern has been observed in previous hamster studies conducted in our laboratory (Wehner et al., 1975a,b; 1976a,b; 1977a,b; 1978). However, a minimum in the MBWs of both NEFA-exposed groups occurred between 250 and 350 days of exposure. A less pronounced and earlier occurring minimum in the MBWs of both the FA-exposed group and the controls is apparent between 90 and 140 days of exposure. There is a close similarity between the MBW curves of the two NEFA-exposed groups, and between the FA-exposed group and the controls (Figure 24). It will be recalled that the two NEFA-exposed groups were housed in one room and the FA-exposed group and controls in an adjacent identical room. Environmental conditions in both rooms were very similar. It thus appears unlikely that factors related to housing might have caused, or contributed to, the minima of the MBW curves.

Mortality

Mortality as a function of time and treatment is shown for each of the four groups in Figures 25 through 28. For convenient comparison the four mortality curves are shown together in Figure 29.

The survival of the four groups of hamsters was compared using both Breslow's statistic (Breslow, 1970) and the Mantel-Cox statistic (Mantel and Haenszel, 1959; Cox, 1972). Neither statistic was significant. The mortality functions were estimated using the Kaplan-Meier (1958) product limit estimate. Several survival statistics are shown in Table 9.

Mortality patterns relative to the cage compartment positions of the animals are presented for each group in Figures 30 through 33. There is apparently no relationship between mortality and position of the animals in the cages.

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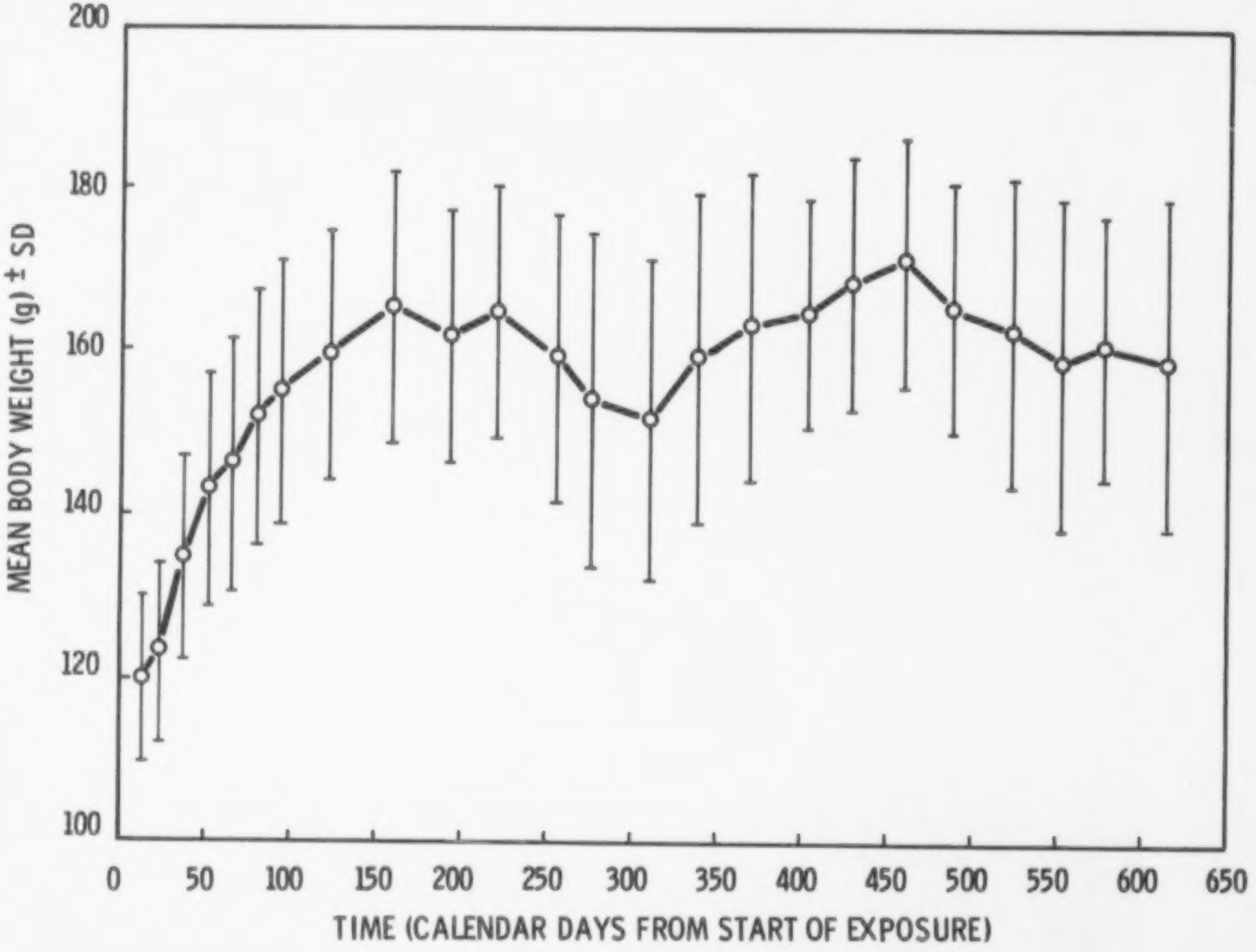

FIGURE 20. Mean body weight of high NEFA-exposed hamsters as a function of time and treatment.

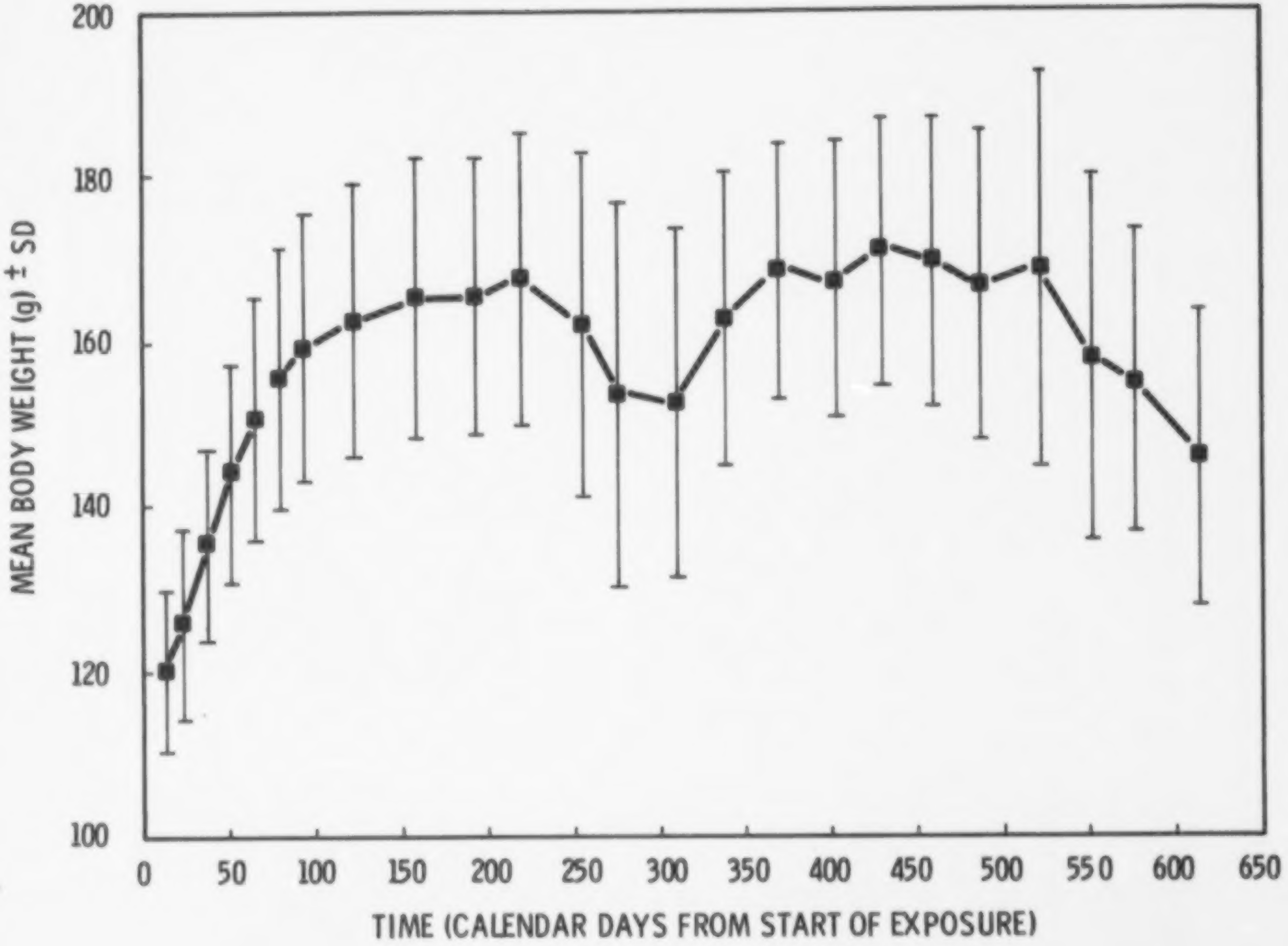

FIGURE 21. Mean body weight of low NEFA-exposed hamsters as a function of time and treatment.

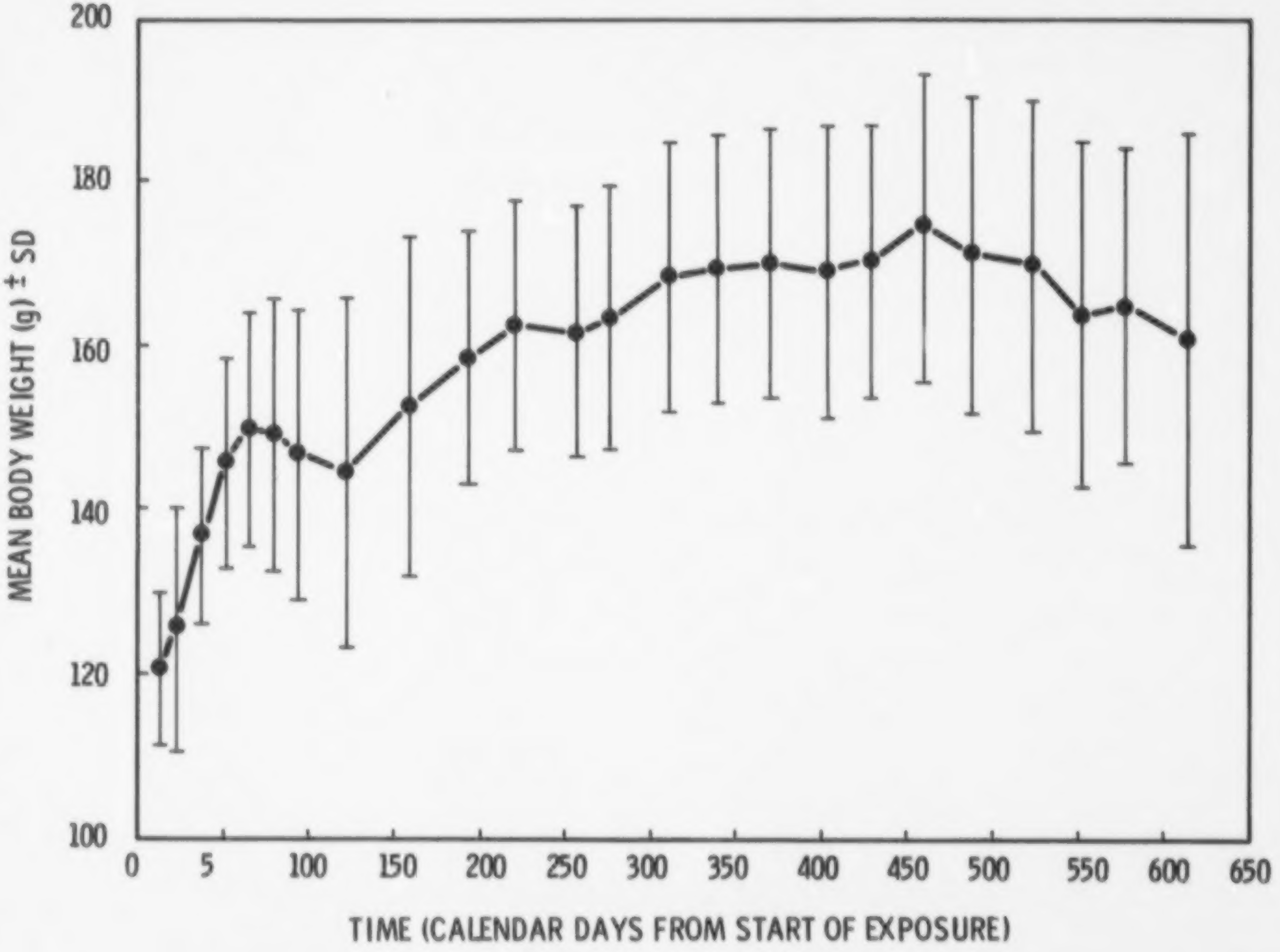

FIGURE 22. Mean body weight of FA-exposed hamsters as a function of time and treatment.

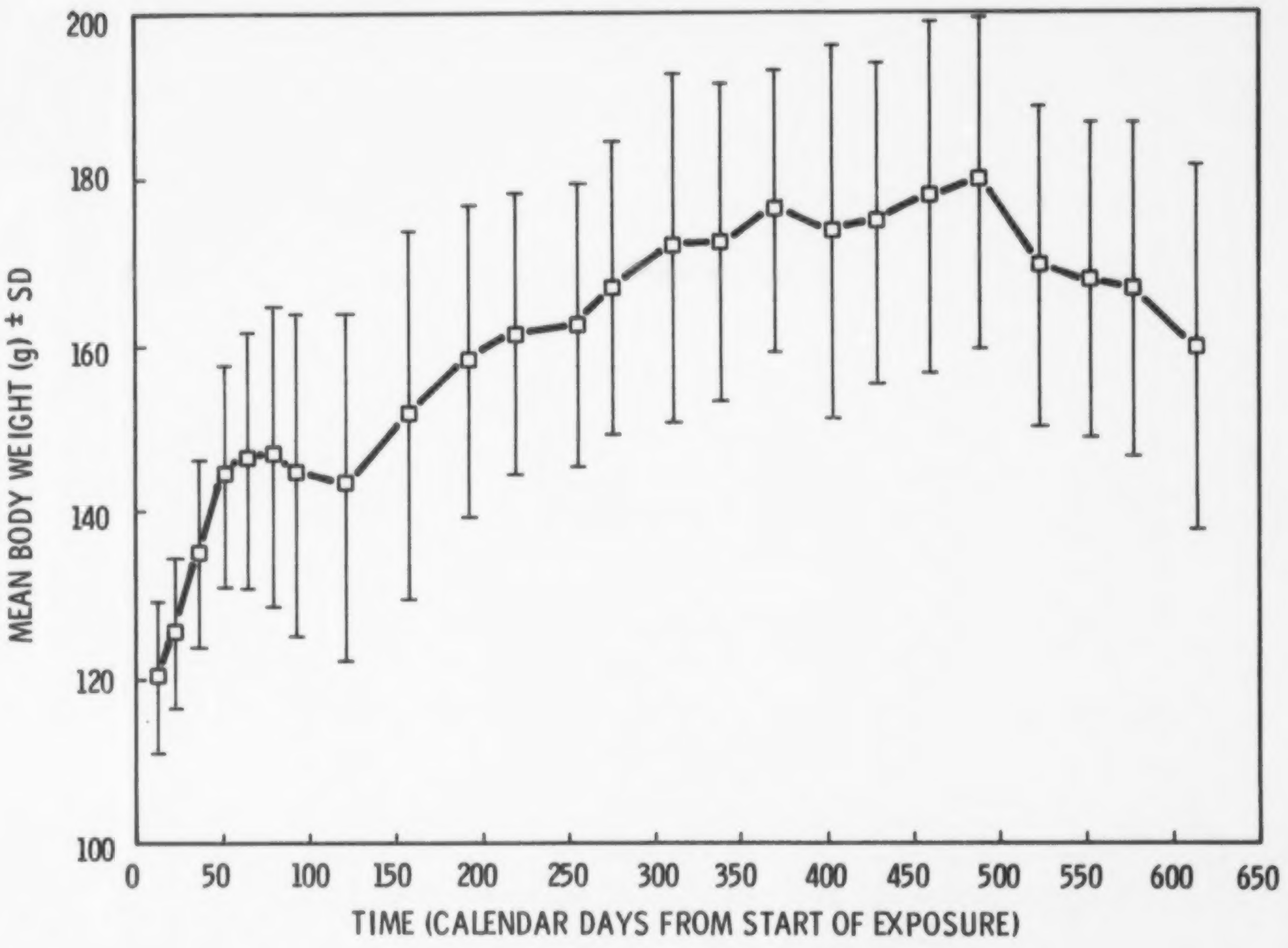

FIGURE 23. Mean body weight of sham-exposed hamters as a function of time and treatment.

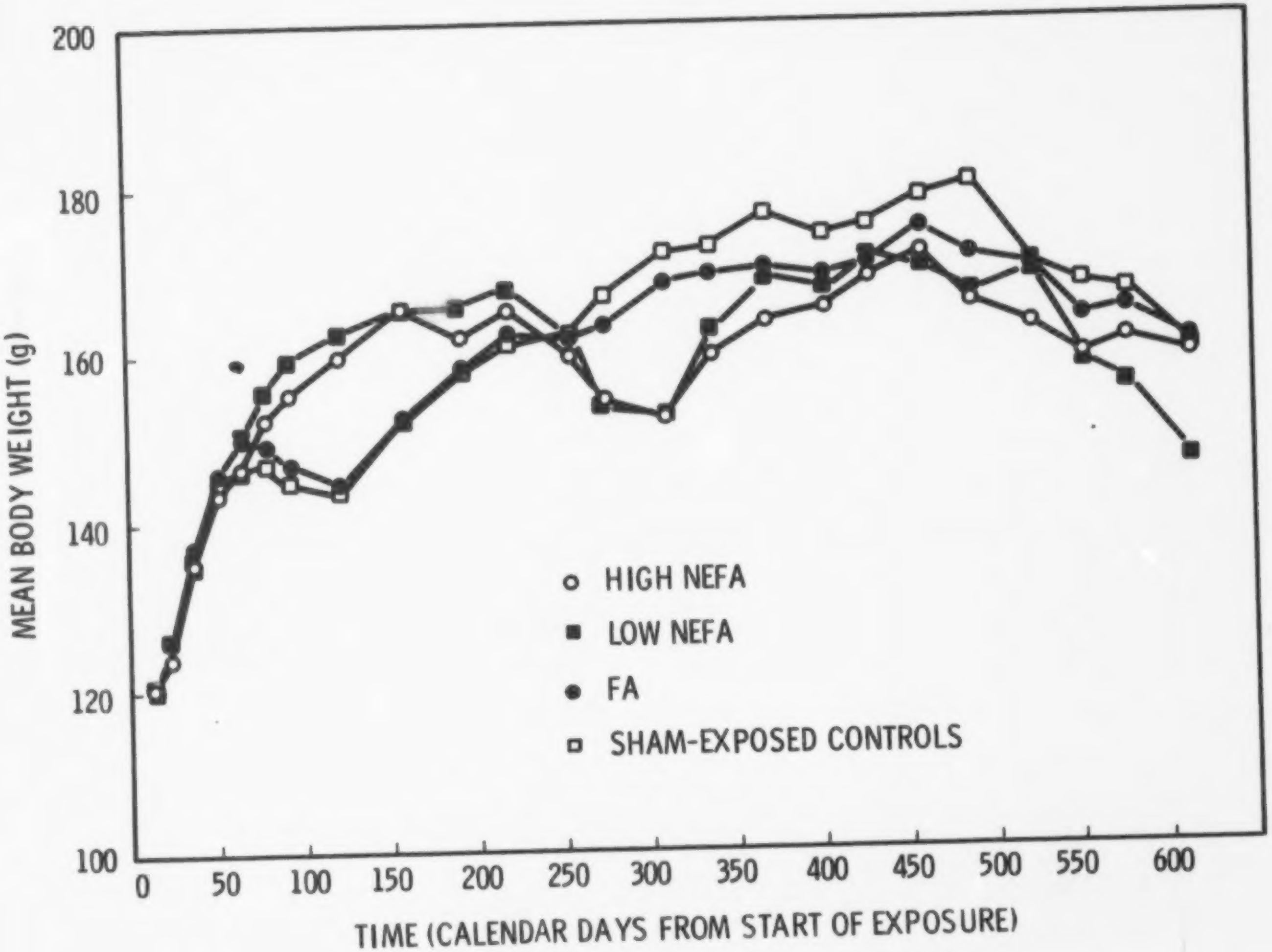

FIGURE 24. Mean body weights of all four hamster groups as a function of time and treatment.

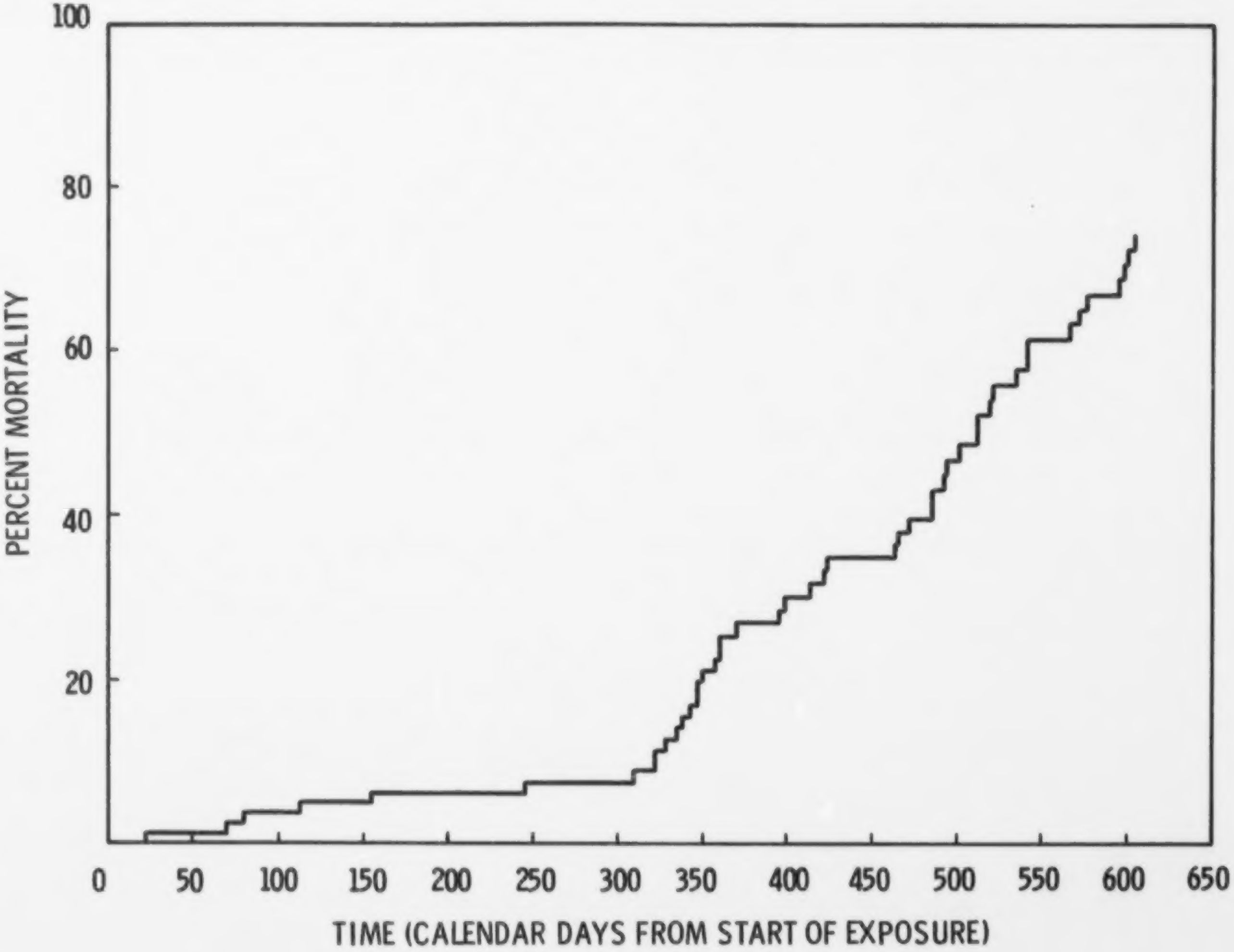

FIGURE 25. Mortality of the high NEFA exposure group as a function of time and treatment.

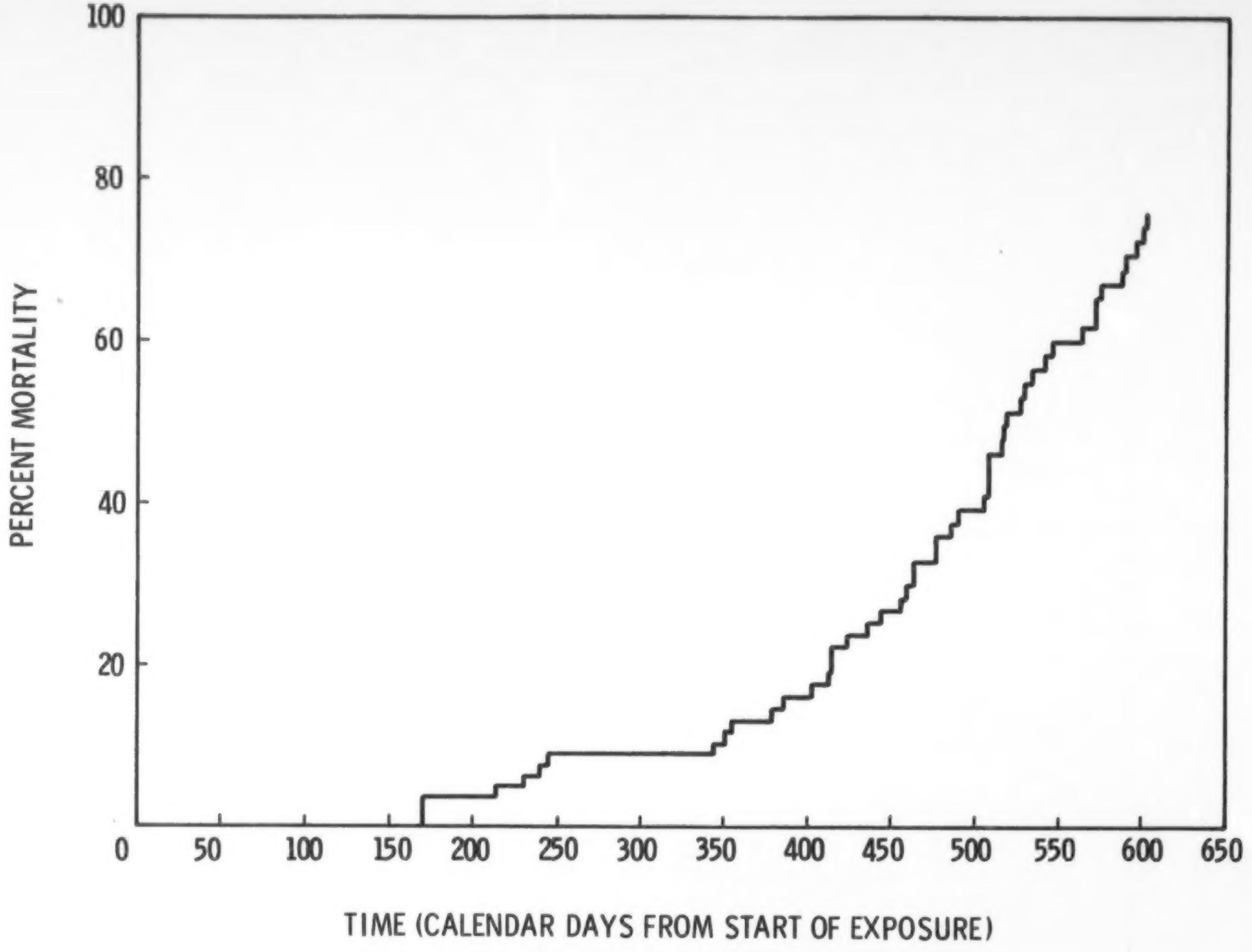

FIGURE 26. Mortality of the low NEFA exposure group as a function of time and treatment.

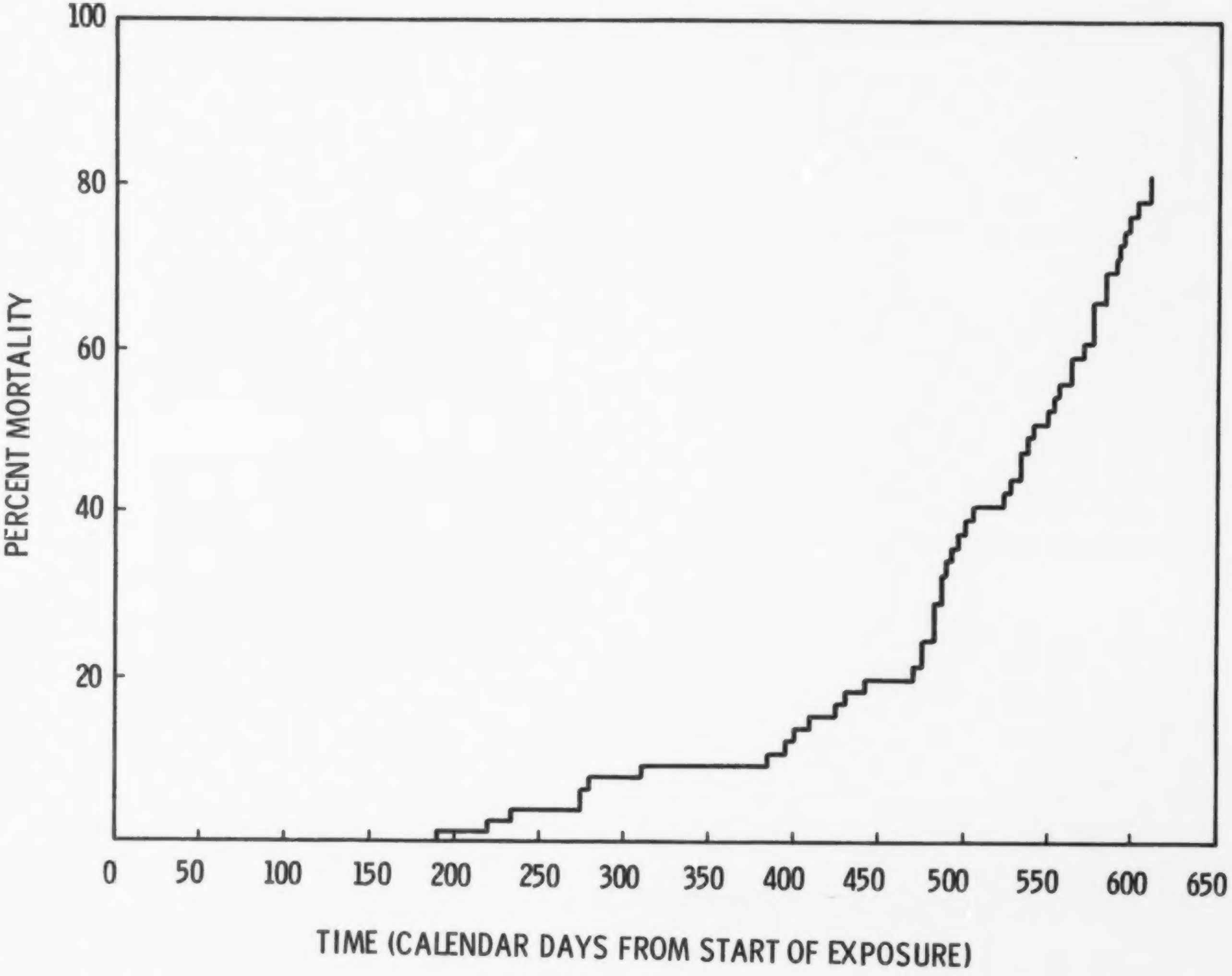

FIGURE 27. Mortality of the FA exposure group as a function of time and treatment.

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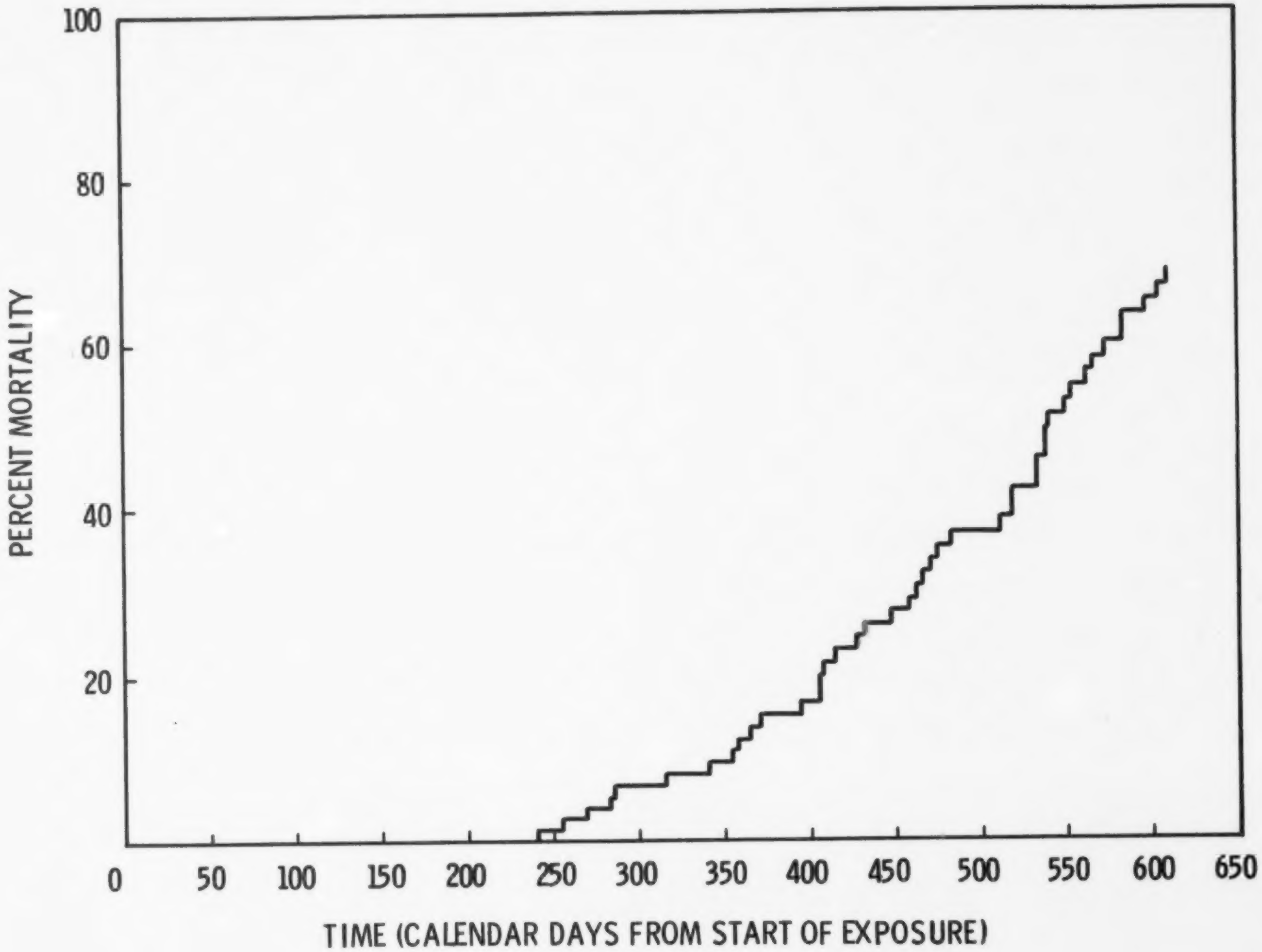

FIGURE 28. Mortality of the sham-exposed control group as a function of time and treatment.

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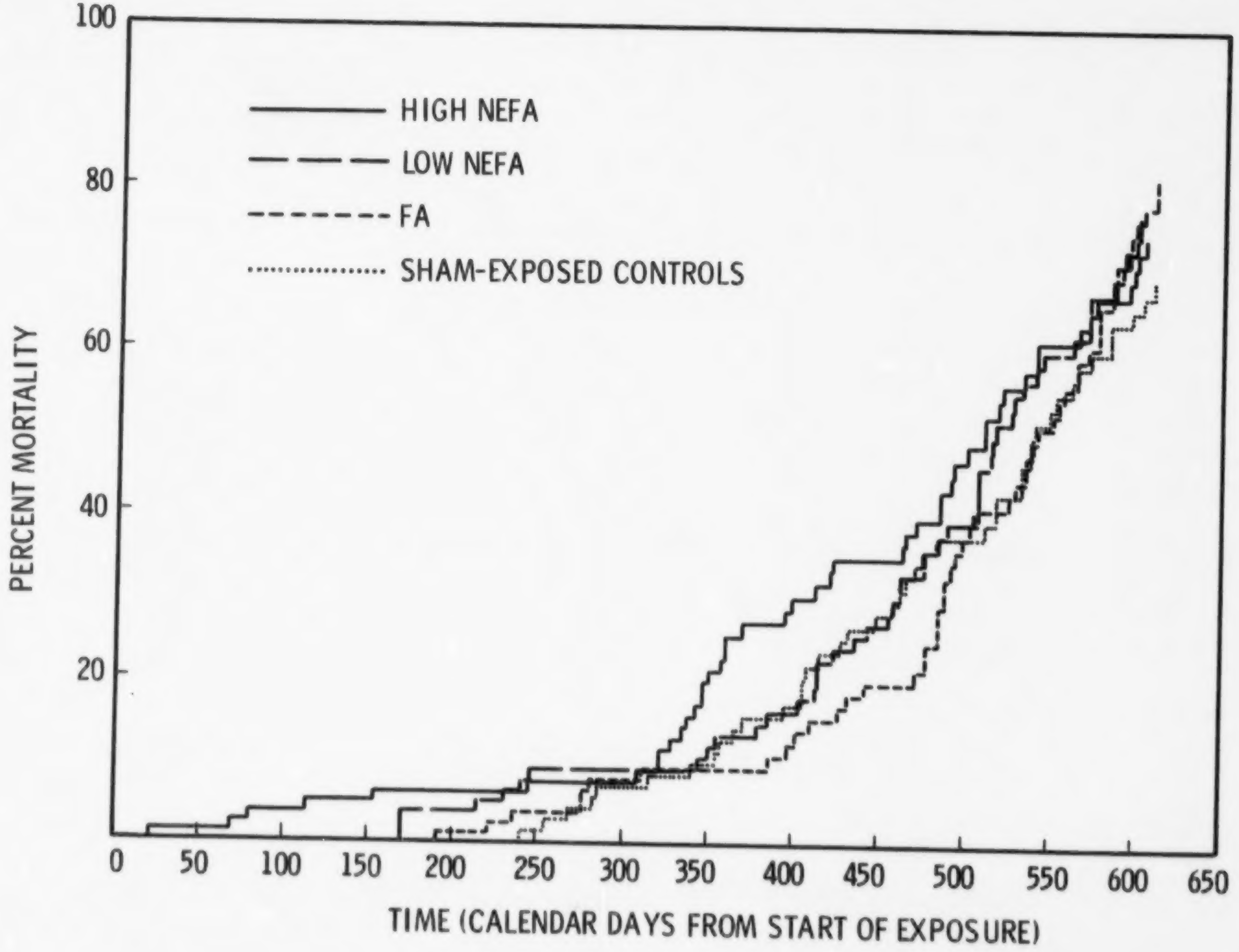

FIGURE 29. Mortality of the four experimental groups as a function of time and treatment.

TABLE 9. SURVIVAL DATA

Group	Mean Survival Time (days)	Median Survival Time (days)	25th Percentile (days)
High NEFA	474*	511	360
Low NEFA	495**	518	436
FA	513†	541	483
Sham-exposed controls	511††	540	432

Breslow test $\chi^2 = 3.667$, $P = 0.30$

Mantel-Cox $\chi^2 = 1.637$, $P = 0.65$

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* Based on maximum survival time of 613 days; SEM 18.26
** Based on maximum survival time of 613 days; SEM 15.47
† Based on maximum survival time of 611 days; SEM 14.01
†† Based on maximum survival time of 612 days; SEM 13.87

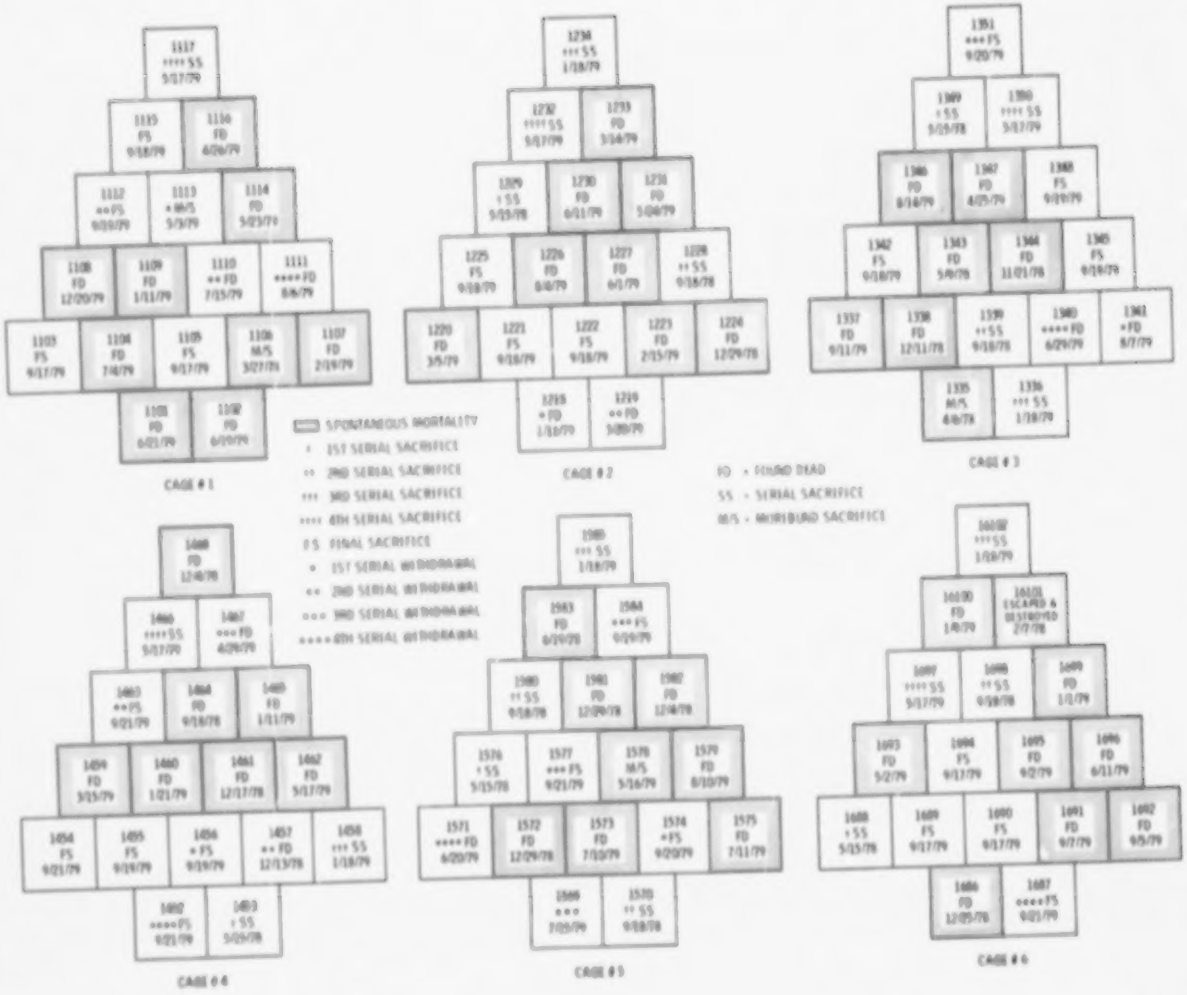

FIGURE 30. Mortality pattern of the hamsters in the high NEFA group.

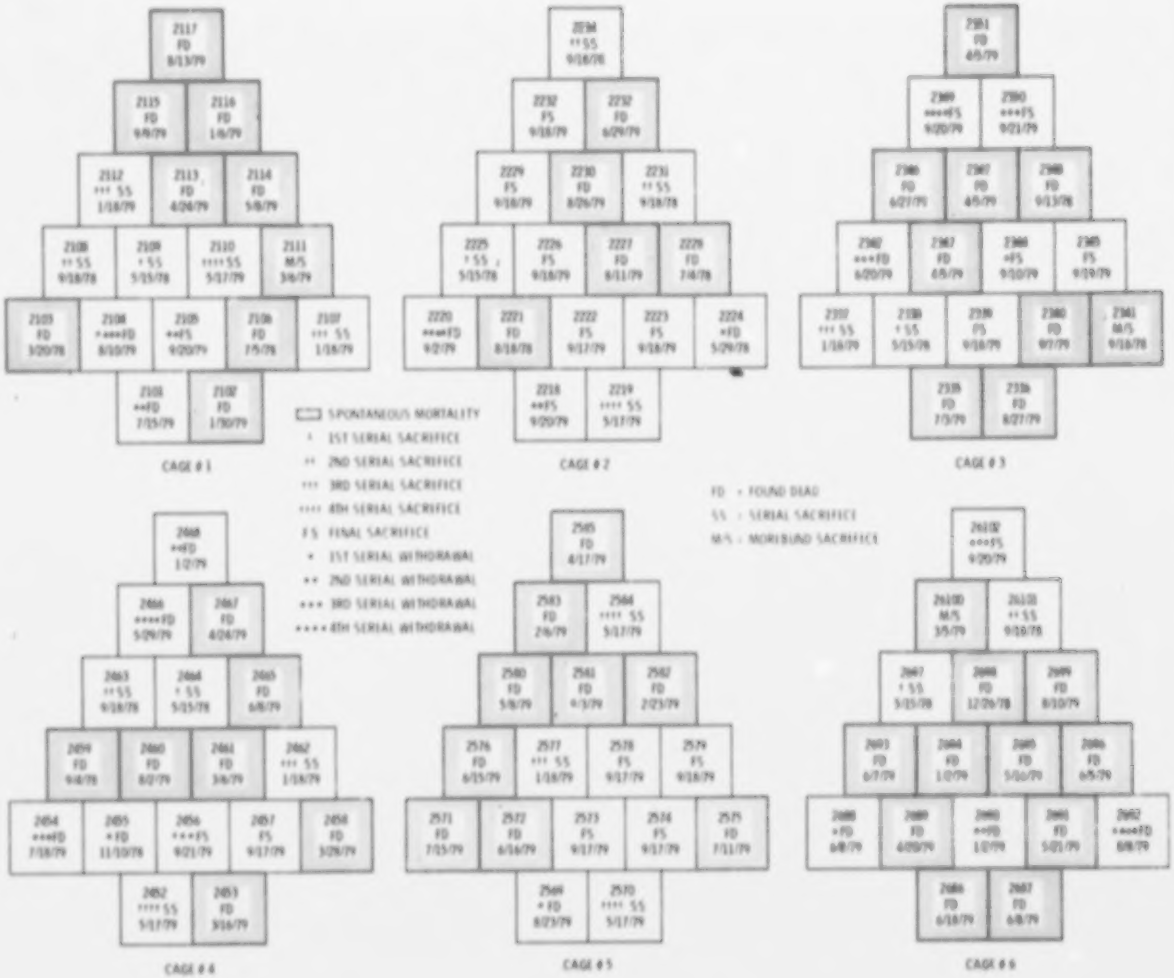

FIGURE 31. Mortality pattern of the hamsters in the low NEFA group.

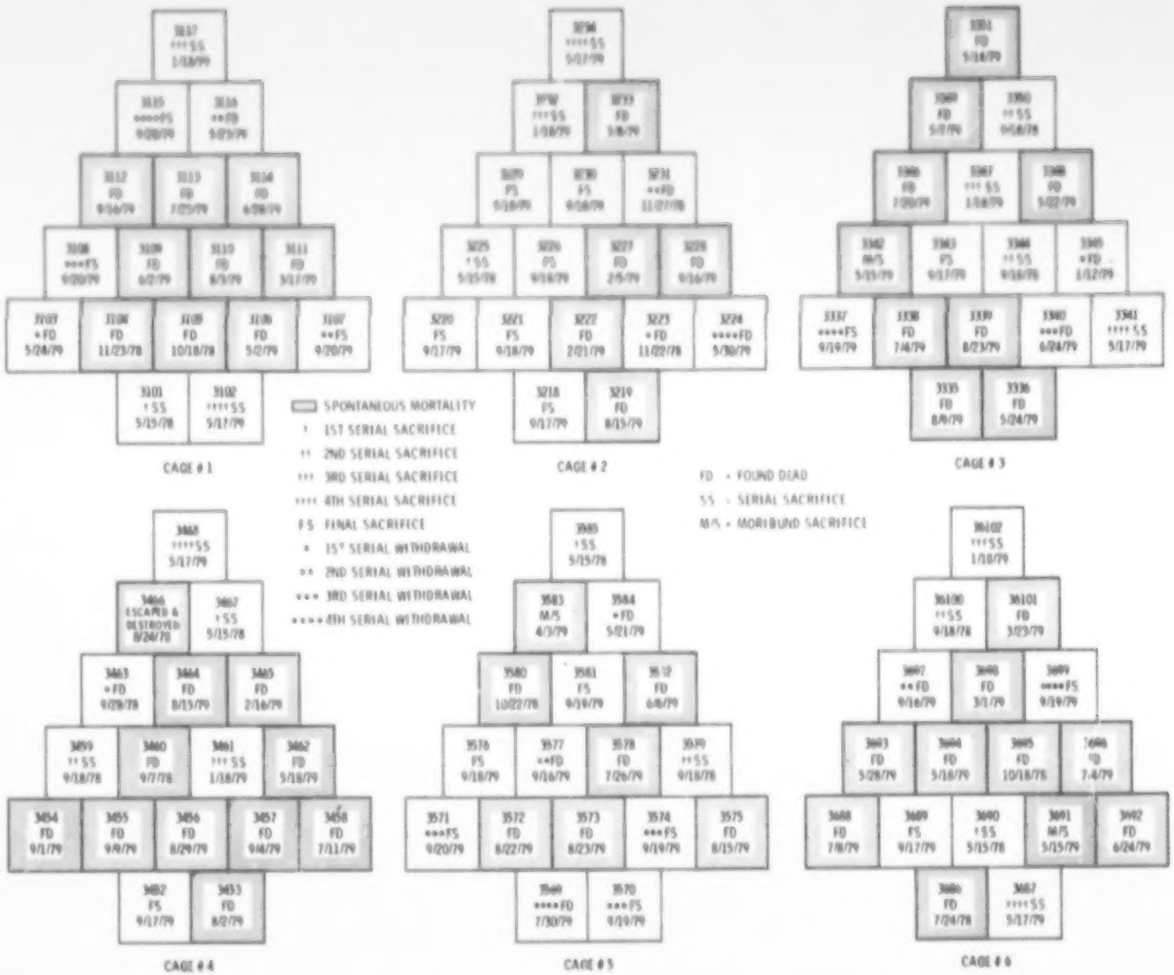

FIGURE 32. Mortality pattern of the hamsters in the FA group.

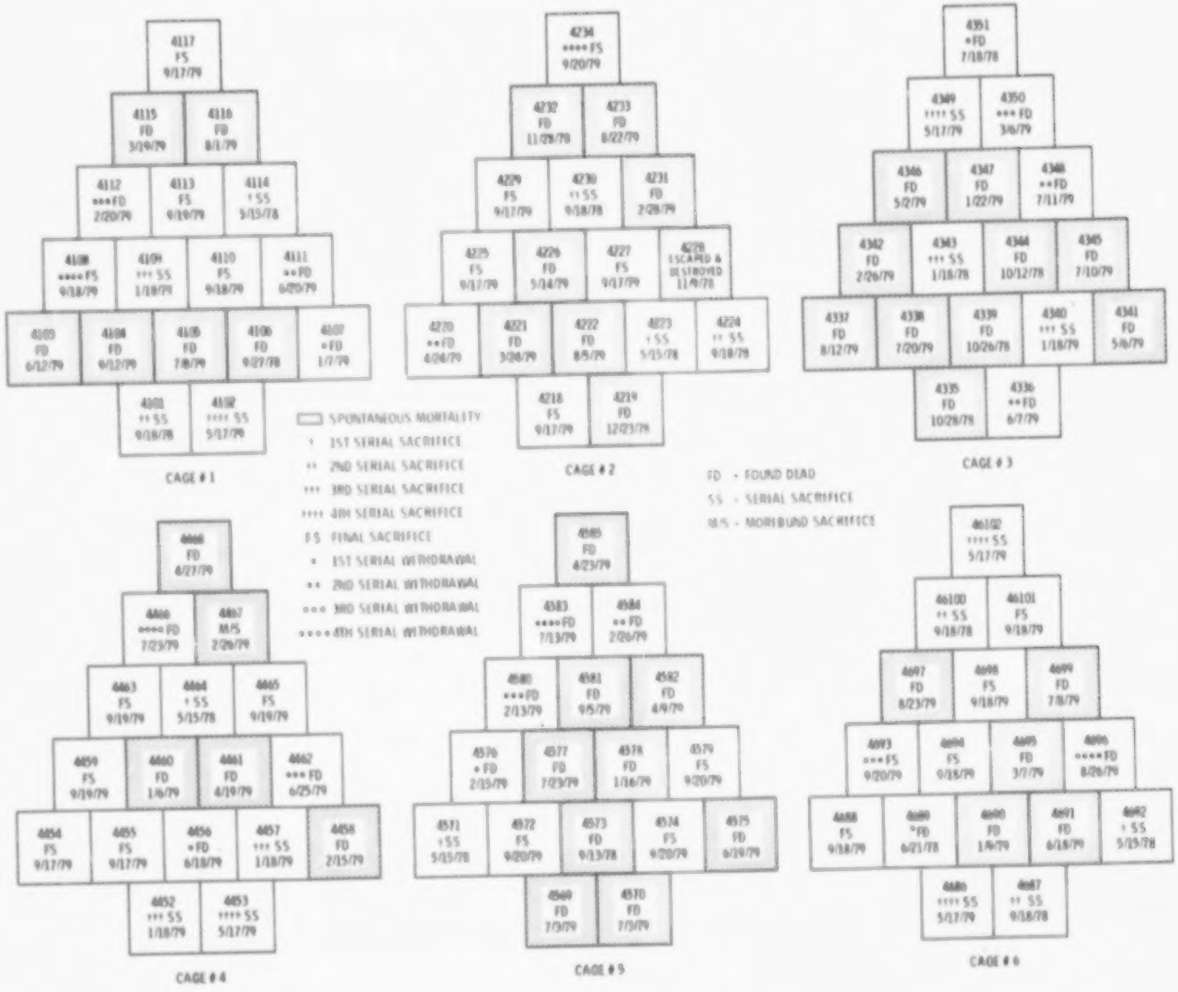

FIGURE 33. Mortality pattern of the hamsters in the sham-exposed control group.

Pathologic Findings

At gross necropsy the lungs of all fly ash-exposed hamsters were darker than the normal, pink lungs of the sham-exposed hamsters. The fly ash-exposed hamster lungs were diffusely tan to grey with black foci, typically 1 to 2 mm in diameter, that tended to be distributed in subpleural areas. The low NEFA-exposed hamsters were less severely involved than the high NEFA or FA-exposed animals at all sacrifice times. At the 4- and 8-month sacrifice times no differences were noted between the high NEFA- and FA-exposed animals. At the 12-and 16-month sacrifice times, the discoloration of the lungs appeared to be more homogeneously distributed in the high NEFA-exposed hamsters as compared to a more focal distribution in the FA-exposed hamsters (Figure 34). However, at the final sacrifice time (20 months of exposure) it was no longer possible to differentiate between the lungs of the high NEFA-and the FA-exposed hamsters. The discoloration of the lungs did not appear to progress in severity after the first year of exposure. There appeared to be a trend towards diminution of discoloration in retired animals.

Group mean body weights, lung weights, and lung volumes from the sacrificed hamsters are tabulated in Table 10. There were no significant differences in body weight among exposure groups at any sacrifice time. The mean lung weights and lung volumes--both absolute and adjusted for body weight--of the high NEFA- and the FA-exposed hamsters were consistently heavier than those of the low NEFA and sham-exposed groups. There were no significant differences in group mean lung weights and volumes between the high NEFA and FA exposure groups, or between the low NEFA and sham-exposed groups.

Histopathologic examination of lung sections showed fly ash dust aggregated in alveoli (SNOP-coded and referred to in the following as anthracosis) throughout the lobes with a predilection for peribronchiolar and subpleural areas (Figure 35). The dust was generally phagocytosed by alveolar macrophages. An interstitial reaction, composed of alveolar septa thickened with prominent alveolar epithelial cells and collagenous stroma (Figure 36) was associated with the dust accumulation. Bronchiolization, composed of proliferation of bronchiolar epithelium into alveolar ducts, was more pronounced in areas of peribronchiolar dust accumulation (Figure 37). Vesicular emphysema, composed of dilated air spaces distal to terminal bronchioles (Figure 38), occasionally occurred, primarily in the final sacrifice (20-month) exposure group (Table 11).

Anthracosis, interstitial reaction, and bronchiolization in the lungs of high NEFA- and FA-exposed hamsters were consistently higher than in low NEFA-exposed hamsters (Table 11). The high NEFA-exposed hamsters had borderline ($P < 0.06$) increased incidence of emphysema compared to the sham-exposed hamsters, and a borderline ($P < 0.06$) increase in severity of anthracosis compared to the FA-exposed hamsters.

The effects of duration of exposure and recovery were assessed by regressing lesion grade on duration of exposure and on the survival time after withdrawal. The regression coefficients are presented in Table 12. Anthracosis progressed in severity with time and decreased with recovery in all exposure groups (high NEFA, low NEFA, and FA). The interstitial reaction progressed in severity with time in the high NEFA- and FA-exposed

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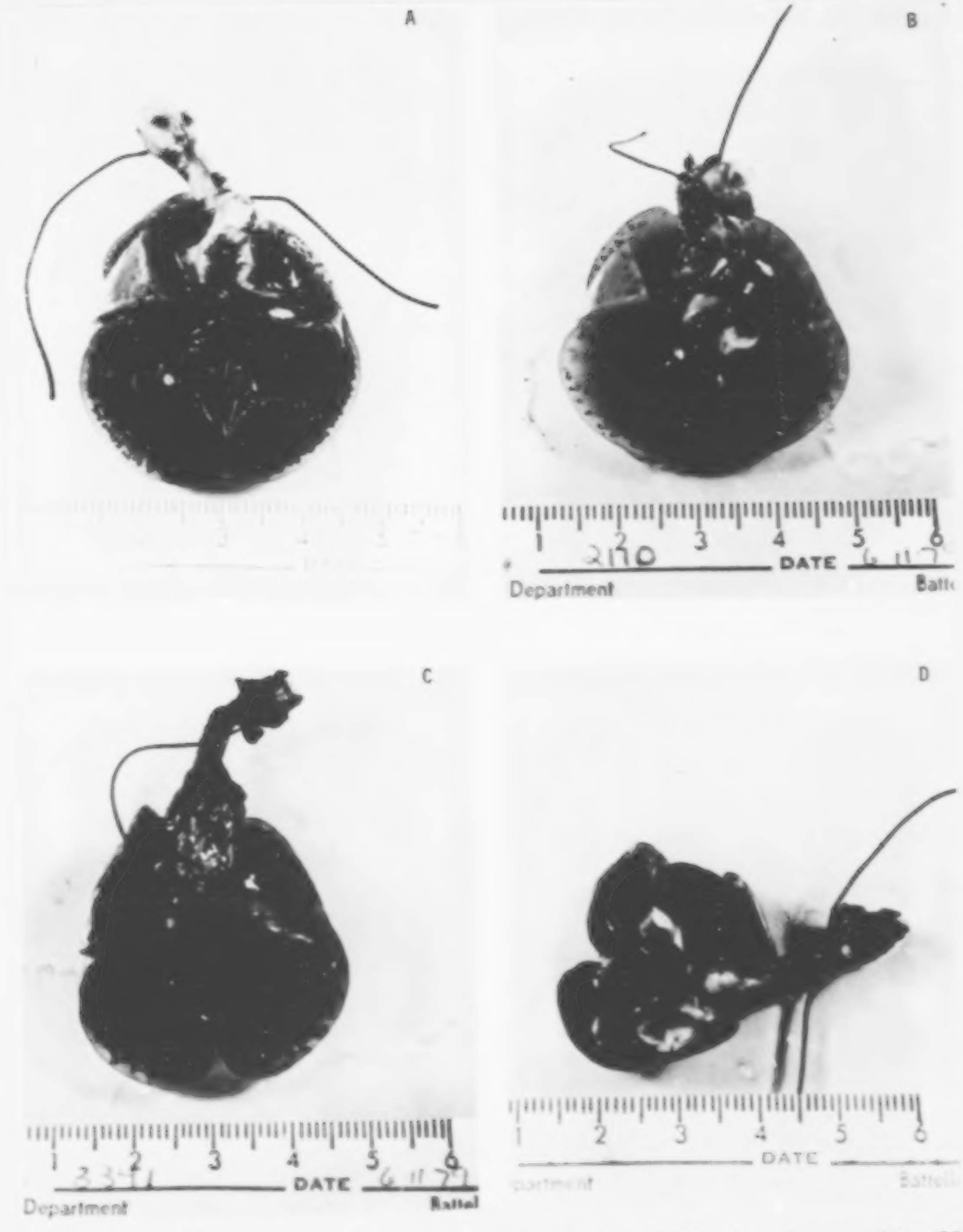

FIGURE 34. Typical gross appearance of lungs from following groups after 16 months of exposure: A = high NEFA; B = low NEFA; C = FA; D = sham-exposed controls.

TABLE 10. BODY WEIGHT, LUNG WEIGHT, AND LUNG VOLUME DATA (MEANS ± SD)

	EXPOSURE GROUP	SERIAL SACRIFICE INTERVALS				
		4 MONTHS*	8 MONTHS*	12 MONTHS*	16 MONTHS*	20 MONTHS*
Body Weight (g)	High NEFA	155 ± 9	169 ± 12	153 ± 23	159 ± 12	150 ± 15
	Low NEFA	168 ± 20	165 ± 28	171 ± 14	158 ± 5	148 ± 19
	FA	147 ± 20	169 ± 12	164 ± 5	170 ± 11	155 ± 16
	SHAM	153 ± 14	155 ± 13	177 ± 12	164 ± 43	150 ± 21
Lung Weight (g)	High NEFA†	1.925 ± 0.20	2.034 ± 0.14	2.672 ± 0.18	2.559 ± 0.13	2.385 ± 0.61
	Low NEFA	1.776 ± 0.24	1.425 ± 0.34	2.394 ± 0.05	1.889 ± 0.18	1.881 ± 0.48
	FA†	1.826 ± 0.10	1.992 ± 0.15	2.480 ± 0.11	2.634 ± 0.41	2.385 ± 0.58
	SHAM	1.708 ± 0.17	1.602 ± 0.47	2.049 ± 0.25	2.114 ± 0.20	2.031 ± 0.53
Lung Weight as % of Body Weight	High NEFA†	1.23 ± 0.06	1.22 ± 0.16	1.77 ± 0.24	1.61 ± 0.08	1.58 ± 0.43
	Low NEFA	1.06 ± 0.04	0.86 ± 0.14	1.41 ± 0.13	1.19 ± 0.11	1.29 ± 0.43
	FA†	1.26 ± 0.17	1.18 ± 0.11	1.51 ± 0.06	1.55 ± 0.20	1.55 ± 0.39
	SHAM	1.12 ± 0.10	1.02 ± 0.23	1.16 ± 0.11	1.35 ± 0.32	1.36 ± 0.42
Lung Volume (mℓ)	High NEFA†	6.47 ± 1.3	6.90 ± 1.0	7.73 ± 0.5	8.30 ± 0.4	6.71 ± 1.0
	Low NEFA	5.55 ± 1.3	6.00 ± 1.1	6.99 ± 0.7	7.40 ± 0.1	5.94 ± 1.2
	FA†	6.25 ± 0.9	7.80 ± 0.4	6.85 ± 0.5	7.70 ± 1.0	6.40 ± 1.1
	SHAM	4.77 ± 0.5	7.39 ± 0.8	6.39 ± 0.8	5.67 ± 0.8	6.46 ± 0.9

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* Time in months since start of exposures at which time the hamsters were 2 months old.

† Significantly different (P <0.01) from Low NEFA and SHAM values (analysis of variance, F-test).

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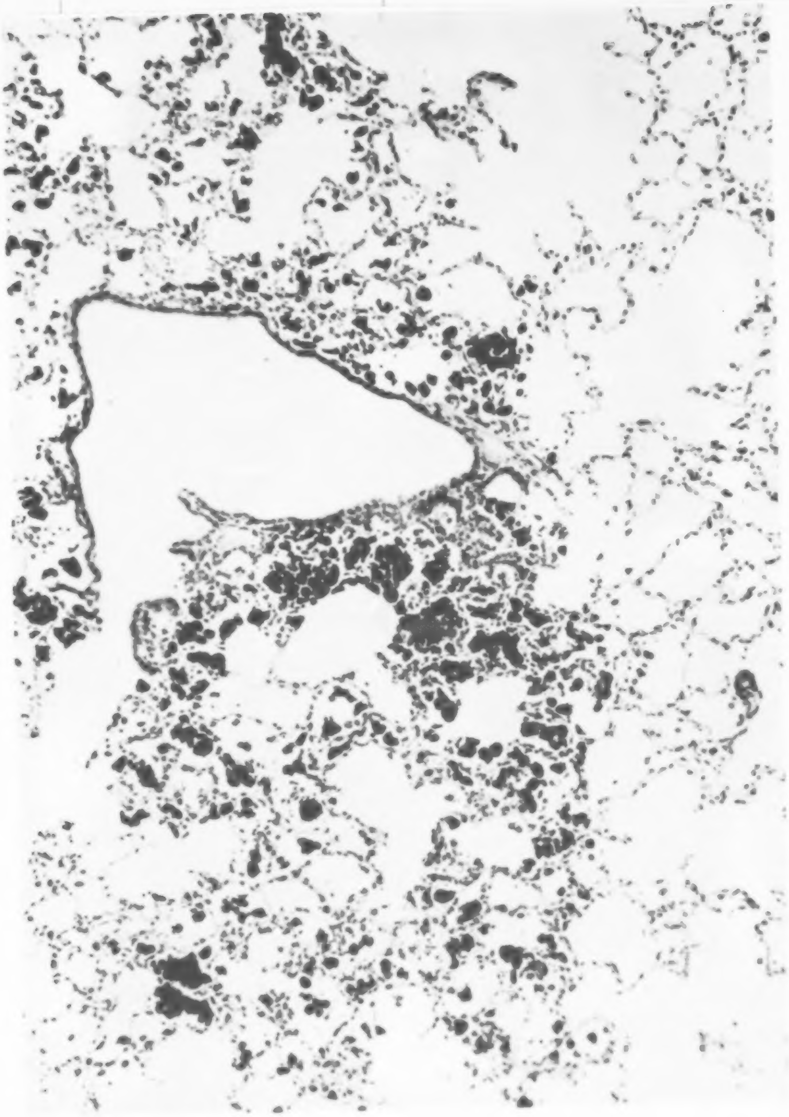

FIGURE 35. Fly ash deposition in lung of hamster #1689, sacrificed after 20 months of exposure to NEFA aerosol (conc. ∿70 μg/ℓ), H&E stain, 125 X.

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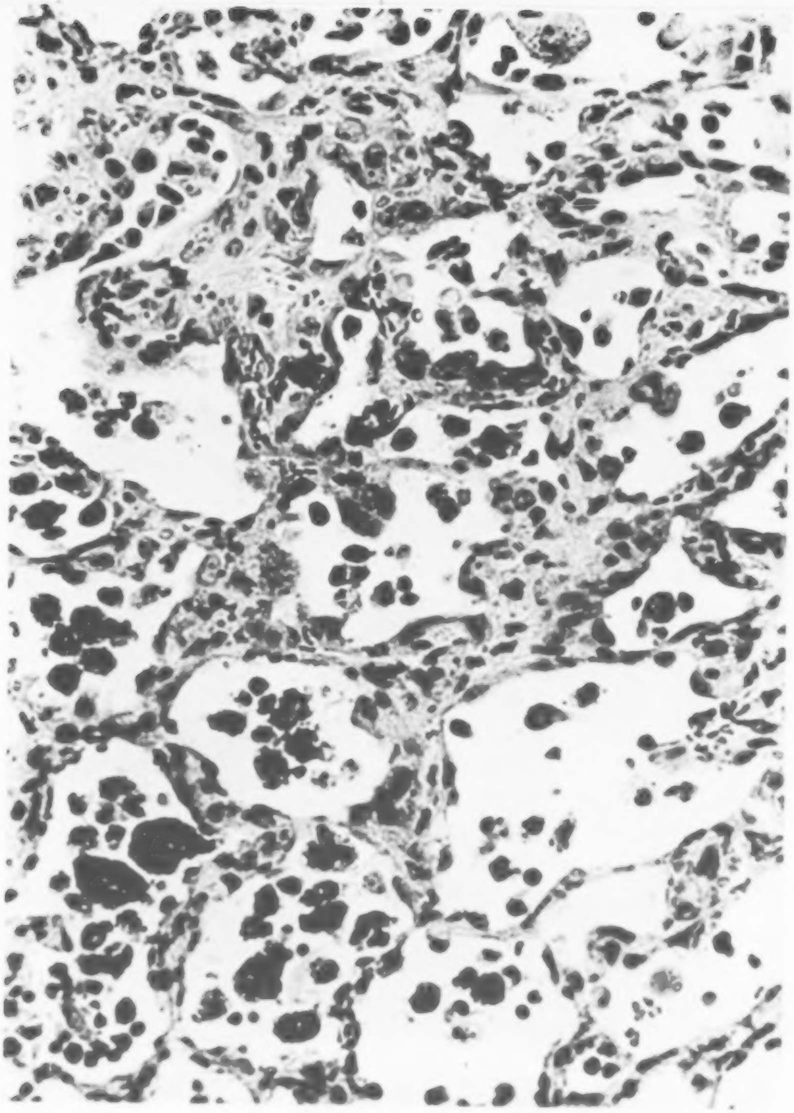

FIGURE 36. Lung of hamster #1348, sacrificed after 20 months of exposure to NEFA aerosol (conc. ~70 μg/ℓ), showing interstitial reaction. H&E stain, 400 X.

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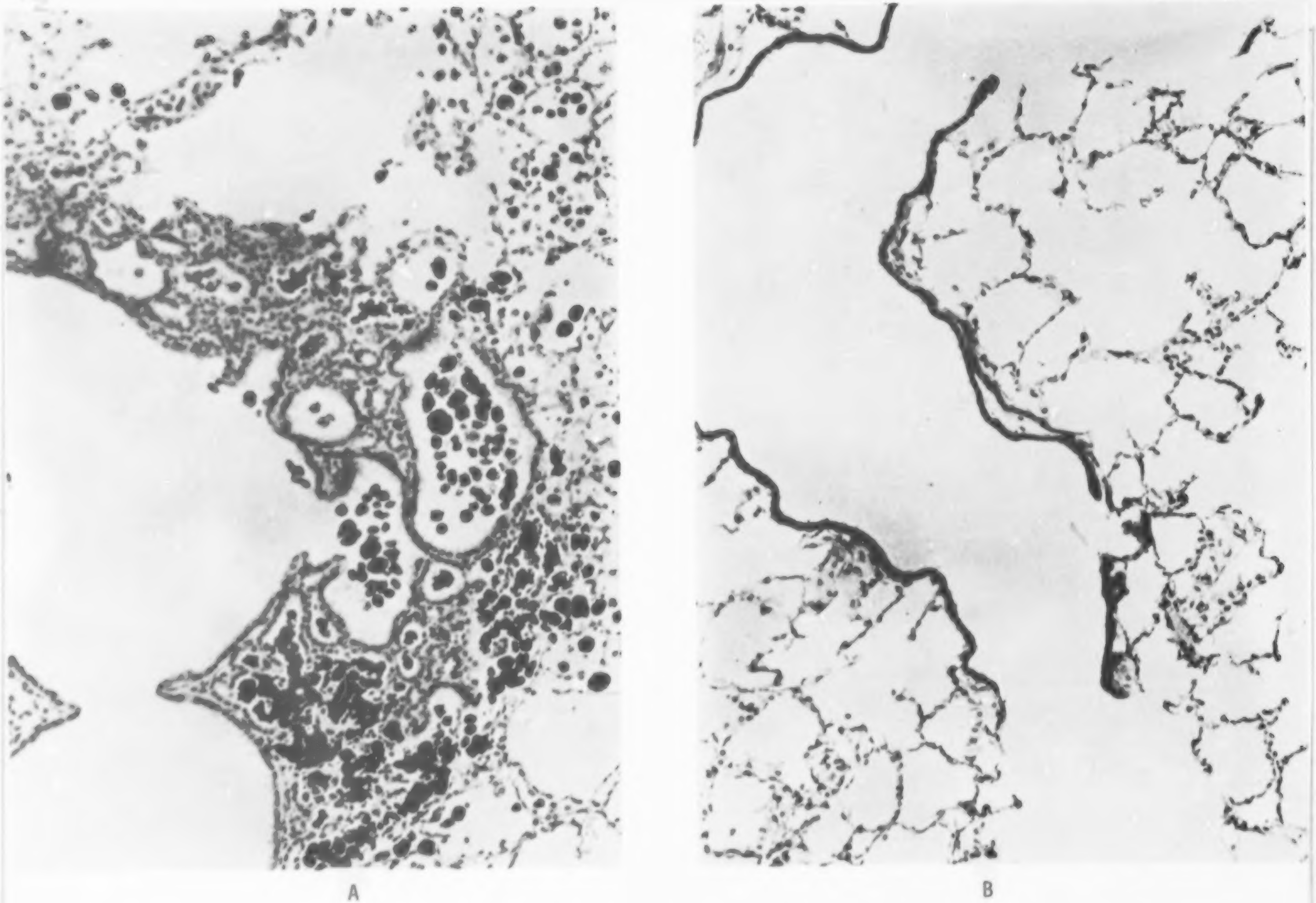

FIGURE 37. Bronchiolization (A) in the lung of the same hamster shown in Figure 36, and lung of control hamster #4113 (B) of the same age (22 months). H&E stains, both 160 X.

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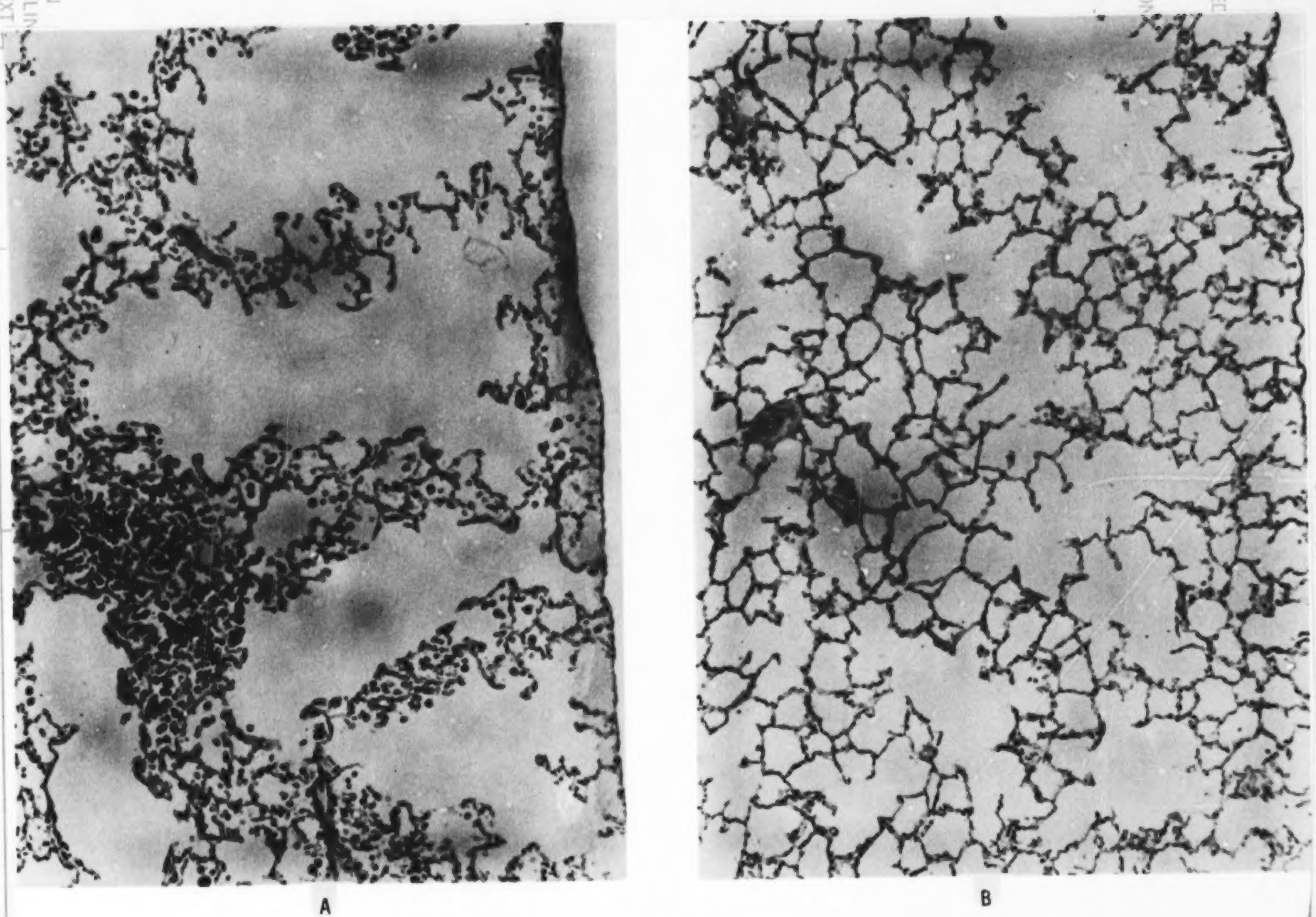

FIGURE 38. Vesicular emphysema (A) in the lung of the same hamster shown in Figure 36(A) and 37(A), and the lung of the same control hamster (B) shown in Figure 37(B). H&E stains, both 100 X.

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TABLE 11. HISTOLOGIC CHANGES RELATED TO EXPOSURE

Lung Lesions	High NEFA			Low NEFA			FA			Sham-Exposed Controls		
	N_1	N_2	G	N_1	N_2	G	N_1	N_2	G	N_1	N_2	G
Anthracosis	61	61	3.41**†	62	62	2.29**††	61	61	3.25**	55	0	--
Interstitial Reaction	61	57	2.23**	62	56	1.39**††	61	61	2.23**	55	4	1.0
Bronchiolization	61	45	1.31**	62	29	1.17*††	61	44	1.45**	55	11	1.18
Emphysema	61	6	1.67†††	62	1	3	61	3	1.67	55	1	2.0

N_1 = Number of animals that died or were killed at final sacrifice time (excluding animals autolysed, periodically sacrificed, or withdrawn from exposure)

N_2 = Number of lesions

G = Average grade

* Significantly different from sham-exposed controls, $P < 0.05$

** Significantly different from sham-exposed controls, $P < 0.01$

† Significantly different from FA-exposed hamsters, $P < 0.06$

†† Significantly different from high NEFA and FA-exposed hamsters, $P < 0.01$

††† Significantly different from sham-exposed hamsters, $P < 0.06$

(Significance tests used Wilcoxon rank-sum statistic. M. Hollander and D. A. Wolfe, Nonparametric Statistical Methods, Wiley, New York, 1973.)

TABLE 12. HISTOLOGIC CHANGES AS A FUNCTION OF DURATION OF EXPOSURE AND RECOVERY TIME†

Lung Lesions	High NEFA		Low NEFA		FA	
	Progression	Recovery	Progression	Recovery	Progression	Recovery
Anthracosis	0.21 (5.36)*	-0.12 (-2.17)*	0.09 (2.58)*	-0.17 (-3.50)*	0.06 (1.78)*	-0.14 (-2.40)*
Interstitial Reaction	0.24 (6.63)*	-0.04 (-0.87)	0.06 (1.54)	--	0.09 (2.60)*	-0.04 (-0.73)
Bronchiolization	0.17 (3.93)*	0.09 (1.43)	--	--	0.26 (5.05)*	0.15 (1.84)*

---

† Values are slopes of linear least-square (regession) fits to plots of grade of lesions vs. time. Values in parentheses are t-values.

* Indicates significant change of condition (P <0.05, Student's t-test)

groups, but did not diminish to any significant degree in any of the exposure groups after the hamsters were withdrawn from exposure. Bronchiolization progressed only in the high NEFA and FA exposure groups, and progressed (although at a reduced rate) in the FA-exposed hamsters after being withdrawn from exposure.

Histopathologic examination of mediastinal lymph nodes (Figure 39) showed an increase in incidence and mean severity of dust deposition (anthracosis) in the high NEFA- and FA-exposed hamsters relative to the low NEFA-exposed hamsters. Although reactive hyperplasia was occasionally observed in mediastinal lymph nodes of fly ash-exposed hamsters, this condition could not clearly be related to fly ash exposure since reactive hyperplasia also occurred in the mediastinal lymph nodes of sham-exposed hamsters. It should be noted that the mediastinal lymph nodes are rather small in hamsters and our routine sections through the anterior mediastinum occasionally were void of lymphoid tissue. In other hamsters the particular mediastinal lymph nodes examined could have been draining regions other than lung with the fly ash deposition.

There were no other lesions clearly related to fly ash exposure. While not statistically significant, it is of interest to note that the only 2 malignant primary thorax neoplasms (Table 13) occurred in the high NEFA-exposed hamsters. The pulmonary adenocarcinoma was found in a hamster from the final sacrifice (20-month) group and consisted of a subpleural, fairly well-circumscribed mass, 3 mm in diameter, having a bronchiolo-alveolar pattern with a low degree of malignancy (Figure 40). The pulmonary mesothelioma was found in a hamster that died after 5 months of exposure. It consisted of large, poorly differentiated, irregularly shaped masses between the left and the azygos lobe of the lung (Figure 41).

The remaining lesions, listed in Appendix A, were of a nature commonly occurring spontaneously in hamsters.

Table 14 shows the means of the estimated pulmonary nickel burdens for the four hamster groups, the pulmonary nickel ratios of the groups and, for purposes of comparison, the ratios of the respirable aerosol concentrations. The ratio of respirable aerosol concentrations for the high NEFA to low NEFA exposures is approximately 4:1. The ratio of mean estimated quantities of nickel/lung at 20 months for these two exposure groups, however, is 8:1. While the difference in ratios is not statistically significant, on the average twice as much nickel appears to have been retained in the lungs of the high NEFA group than the aerosol data suggest. This observation would suggest that the pulmonary clearance rate was slower in the high NEFA-exposed animals than in the low NEFA-exposed animals. Past experience in our laboratory has shown that pulmonary clearance rates can be a function of aerosol concentration.

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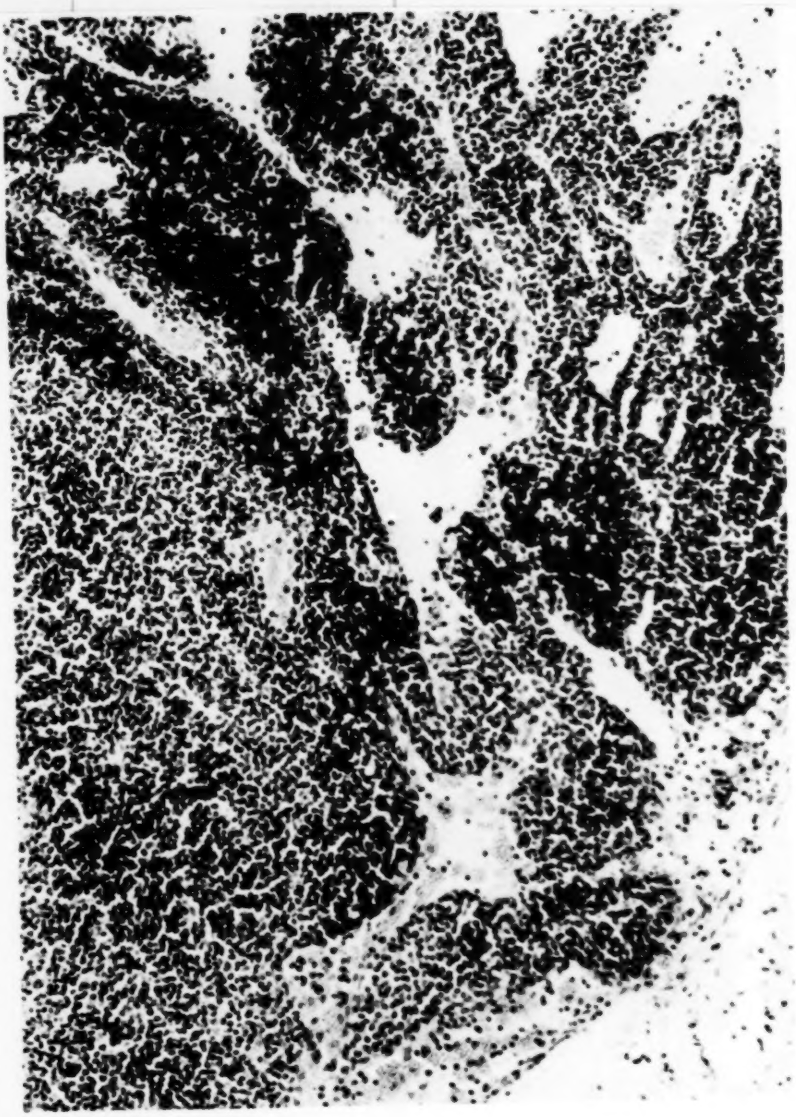

FIGURE 39. Lung of hamster #1115, sacrificed after 20 months of exposure to NEFA aerosol (conc. ∿70 μg/ℓ), showing anterior mediastinal lymph node with NEFA deposition. H&E stain, 160 X.

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TABLE 13. CLASSIFICATION OF NEOPLASMS

Neoplasms	High NEFA	Low NEFA	FA	Sham	Total
Malignant					
Pulmonary Adenocarcinoma	1				1
Pulmonary Mesothelioma	1				1
Mediastinal Hemangiosarcoma			1		1
Splenic Hemangiosarcoma		1			1
Skin Hemangiosarcoma		1*			1
Skin Carcinoma			1*		1
Skin Melanoma				1	1
Malignant Lymphoma				2*	2
Esophageal Fibrosarcoma				1	1
Adrenal Carcinoma	1	2	1*		4
TOTAL MALIGNANT	3	4	3	4	14
Benign					
Laryngeal Polyp		1			1
Adrenal Adenoma	4	10	10	7	31
Pheochromocytoma		1	3	2	6
Pancreatic Islet Adenoma				1	1
TOTAL BENIGN	4	12	13	10	39
TOTAL (MALIGNANT & BENIGN)	7	16	16	14	53

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* Metastases present

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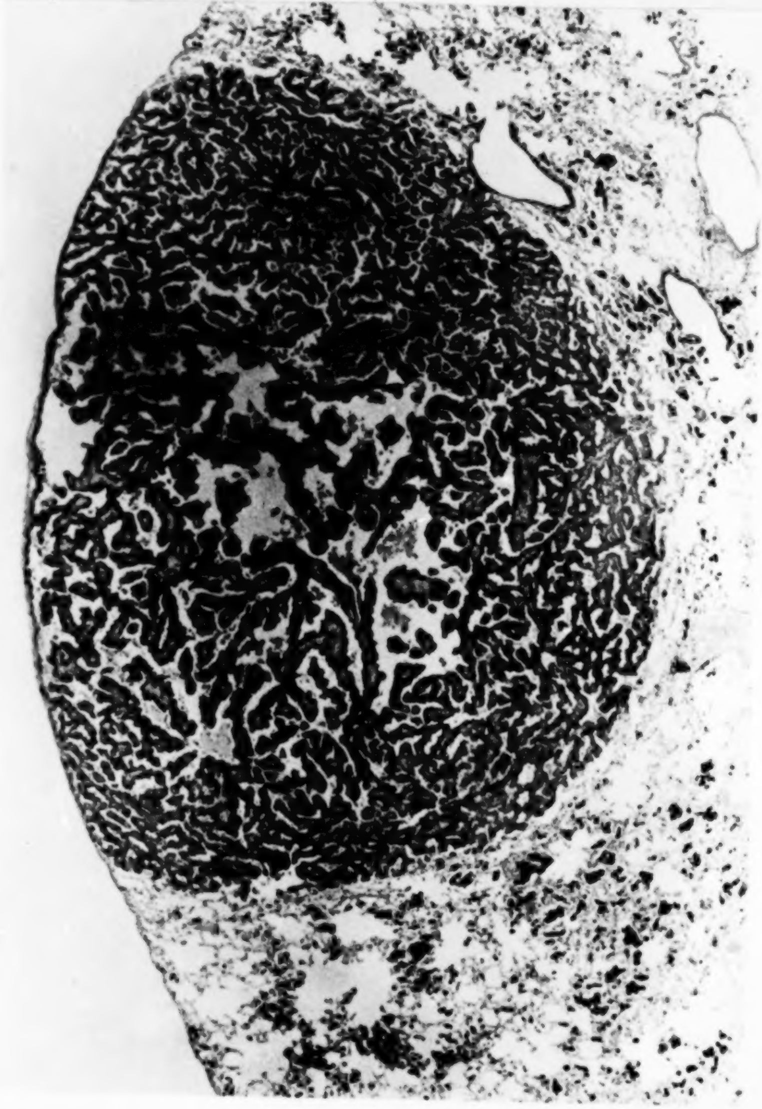

FIGURE 40. Bronchiolo-alveolar carcinoma in lung of hamster #1689, sacrificed after 20 months of exposure to NEFA aerosol (conc. ∿70 μg/ℓ). H&E stain, 31 X.

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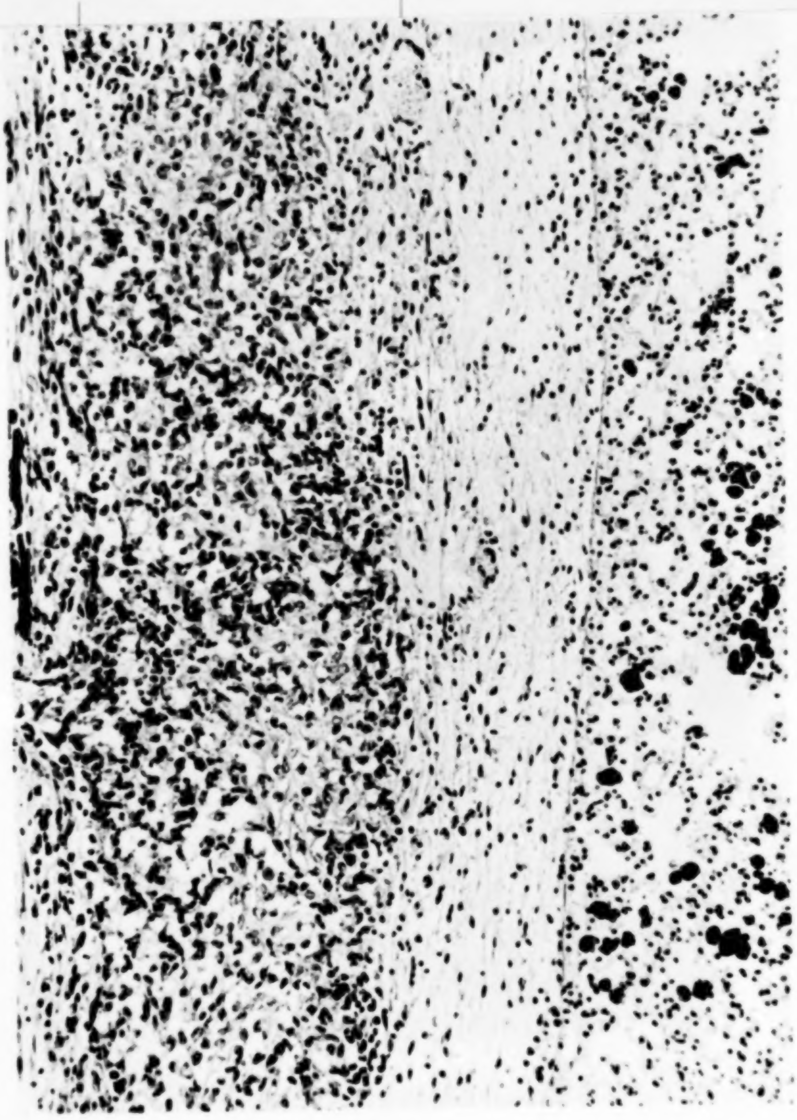

FIGURE 41. Mesothelioma in lung of hamster #1583, found dead after 5 months of exposure to NEFA aerosol (conc. ∿70 μg/ℓ). H&E stain, 160 X.

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TABLE 14. ESTIMATED QUANTITIES OF NICKEL PER LUNG (MEAN ± SD)

Exposure Group	N*	Ni/Lung (μg)**	Pulmonary Ni Ratios of Groups	Ratios of Respirable Aerosol Concentrations
High NEFA (~70 μg/ℓ)†	21	731 ± 507		
			High NEFA/Low NEFA ~8:1	High NEFA/Low NEFA ~4:1
Low NEFA (~17 μg/ℓ)†	22	91 ± 65		
			Low NEFA/FA ~2:1 High NEFA/FA ~17:1	High NEFA/FA = 1:1
FA (~70 μg/ℓ)†	18	42 ± 27		
			FA/Sham ~7:1	
Sham-Exposed Controls	17	6 ± 2.7		

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* N = Number of lungs (known fraction of total lung) analyzed

** Determined by FAAS analysis, estimated for total lung

† Mean respirable aerosol concentration

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DISCUSSION

Past exposures of laboratory animals to low concentrations of fly ash had no or no significant biological effects. Alarie et al. (1973) found no lesions in monkeys and guinea pigs exposed to fly ash aerosol (0.5 μg/ℓ) for up to 78 weeks. MacFarland et al. (1971) exposed cynamolgus monkeys to fly ash (0.16 μg/ℓ and 0.46 μg/ℓ, 23 hrs/day, 7 days/week, for 18 months) and found no effects on lung function, hematological and biochemical parameters. Histologically, pulmonary reaction was "similar to that seen with any 'nuisance' dust and is not considered to be evidence of permanent pathological alteration."

Reviewing the data developed in the course of the studies described in this report, the following picture emerges.

Exposures of hamsters to rather high concentrations of NEFA had no significant effect on the apparent well-being, body weight and life span of the animals although heavy deposits of NEFA in the lungs were demonstrated. The lung weights of the high NEFA- and of the FA-exposed animals, however, were significantly (P <0.01) higher than those of the low NEFA group and the controls. Lung weights, both absolute and adjusted for body weight, might thus be a sensitive indicator of early lung changes and an aid in dose group differentiation in terms of effects of dose levels deposited in, rather than offered to, the animals. There was no significant difference between the MBWs of the high NEFA group and the FA group.

In the chronic exposure experiment the mean lung volumes were significantly (P <0.01) larger for the high NEFA group and the FA group than for the low NEFA group and the controls. Lung volumes were not measured as part of the acute and subchronic exposures. Perhaps lung volumes, too, may be a sensitive indicator of early lung changes, just as suggested above for lung weights.

Histopathologic results of the subchronic exposure showed heavy dust (NEFA or FA) deposition and interstitial reaction in the lungs of all exposed animals, but no significant (P <0.05) differences between the high NEFA- and the FA-exposed animals. The chronic exposures resulted in a 100% incidence of dust deposition, SNOP-coded and referred to in this report as anthracosis, in all dust-exposed groups. In addition, incidence and severity of interstitial reaction and bronchiolization were significantly higher in the dust-exposed groups than in the sham-exposed controls. The only difference between the high NEFA group (70 μg/ℓ NEFA) and the FA group (70 μg/ℓ FA) was an increase in the severity of anthracosis which, however, was only of borderline significance (P <0.06; Table 11). The severity of anthracosis, interstitial reaction and bronchiolization were significantly

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lower ($P < 0.01$) in the low NEFA group (17 μg/ℓ) than in the two other dust-exposed groups (high NEFA, 70 μg/ℓ; FA, 70 μg/ℓ). This dose-effect relationship reflects the two different dose groups, namely low NEFA (17 μg/ℓ) versus high NEFA (70 μg/ℓ) and FA (70 μg/ℓ), but, as stated above, no significant ($P < 0.05$) differences were observed between the high NEFA group and the FA group. While two malignant primary thorax tumors (one pulmonary adenocarcinoma, one pulmonary mesothelioma) were found in two hamsters of the high NEFA group, no statistically significant carcinogenesis was observed.

Of the exposure-related lesions, only anthracosis (dust deposition) decreased as a function of recovery time. This phenomenon was observed in all three dust-exposed groups. Results of the pulmonary deposition, translocation and clearance study with neutron-activated FA (q.v. under ANCILLARY STUDIES, below) support this observation.

The results of our study thus lead to the conclusion that the addition of nickel to fly ash under our experimental conditions did not significantly ($P < 0.05$) enhance the pathogenicity (including carcinogenicity) of fly ash in our animal model. In other words, the quantity of dust--be it NEFA or FA--rather than its Ni content appears to be the major factor in determining tissue response.

While this study was in progress, Hayes and co-workers (Hayes et al., 1978; 1980) developed a technique for examining individual fly ash particles, using scanning electron microscopy (SEM) and energy dispersive X-ray analysis. They found that certain elements in fly ash, including nickel, are very inhomogenously distributed among individual fly ash particles. In other words, a given fly ash, analyzed as containing, say, 0.5% of a certain element, will consist of many particles containing much less or no detectable traces of this element, plus a few particles containing 20%, 30% or even more of this element. Multiple particle (bulk) analysis does not reflect the wide variations in chemical concentration among individual particles to which a cell at the site of impingement of such a particle would be exposed. One can thus be lead to wrong conclusions if one attempts to assess the biological or toxicologic effects of the element in question based on multiple-particle (bulk) analytical procedures. Biological effects of inhaled particulates, including mutagenesis or carcinogenesis, generally originate at the cellular level. The exposure of a cell to an individual particle containing a hundred-fold higher concentration of a potential mutagen or carcinogen than shown by bulk analysis, can be expected to be more hazardous for this cell than the bulk analysis data imply.

We sent a NEFA and a FA sample to Hayes at the Donner Laboratory, University of California, Berkeley, for analysis. Hayes et al. examined 22 particles from the NEFA sample, using their technique (Hayes et al., 1978; 1980). The results (Table 15), while not conclusive on account of the small number of particles analyzed, indicate that the nickel content in 50% of the particles was below the detection limit of their instruments (<0.1% or 1000 ppm); several particles contained from 2 to 4% nickel; one particle contained 28.66% nickel. The nickel content in all examined FA particles was below detection limits (<0.1%). This analysis shows that the nickel was not uniformly distributed in the NEFA particles by the nickel enrichment process,

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TABLE 15. NICKEL CONTENT OF 22 NEFA PARTICLES (%)*

Particle #	Particle Configuration	Diameter (μm)	Ni
1	/	/	1.16
2	S	1.5	28.66
3	NS	3.5	-
4	S	1	-
5	S	2	1.57
6	NS	2	-
7	NS	3	-
8	NS	4	2.10
9	S	2	2.51
10	NS	2.5	-
11	/	/	Yes**
12	NS	/	-
13	S	1	-
14	S	3	0.70
15	S	2	3.62
16	NS	25	-
17	S	2	2.07
18	NS	4	3.69
19	NS	3	-
20	NS	2.5	-
21	S	1	-
22	S	3	0.29

---

* = Analyses courtesy of Dr. Thomas L. Hayes, Donner Laboratory, University of California, Berkeley, using SEM and energy-dispersive X-ray analysis. The nickel content in all FA particles examined by Dr. Hayes was below detection limits (<0.1%).

** = Only qualitative data available

/ = No data available

S = Spherical

NS = Nonspherical

- = <0.1%

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as one might have assumed. Instead, the NEFA particles showed a very heterogeneous distribution of the added nickel that Hayes et al. (1978) found to be typical of certain elements in ordinary fly ash. While the number of particles examined is too small to draw firm or general conclusions, the data in Table 14 suggest no relationship among the 22 particles between nickel concentration and particle size or configuration (spherical or nonspherical).

There are indications that the difference in nickel concentration between the NEFA and the FA was smaller for the chronic study than for the acute and subchronic studies. The first dissolution experiment indicated 0.039% nickel for FA. The second dissolution experiment indicated 0.032% nickel for total FA--which is in reasonable agreement with the 0.039% value--and 0.38% nickel for FA particles smaller than 53 μm (Table 3). The tenfold difference in nickel content might be satisfactorily explained as a function of particle size (Natusch and Wallace, 1974; Campbell et al., 1978; Fisher et al., 1978a). One would then expect a nickel content of at least 0.38% in the respirable particles deposited in the lungs of the FA-exposed hamsters. However, the quantities of nickel in the FA-exposed hamsters of the acute and subchronic experiments were below detection limits. This suggests that, given the quantities of FA deposited in these animals, these respirable FA particles contained substantially less than 0.38% nickel or the nickel would have been detected. This suggestion is supported by the observation that the nickel in the FA sample sent to Hayes (7/5/1978) was below the detection limit of 0.1% for his system.

However, in the chronic exposure experiment the mean quantity of nickel in the lungs of the high NEFA-exposed hamsters was only approximately 20-times higher than that in the FA-exposed hamster lungs, and that in the low NEFA-exposed hamster lungs only about twice that in the FA-exposed hamster lungs, as determined by FAAS after the final sacrifice (Table 14). These analyses, although not part of the experimental protocol, were performed on known quantities of lung tissue to obtain estimates of the total lung burdens in the three dust-exposed hamster groups. The results of these analyses prompted us to reanalyze leftover quantities of FA and NEFA. The reanalyses of remaining quantities of NEFA and FA, collected at different times from different containers (e.g. elutriated material in WDFM cylinders, bulk samples, etc.) showed values ranging from 1.828 to 6.215% nickel for NEFA and 0.296 to 0.605% nickel for FA. The variations in nickel content within the NEFA or FA samples are probably due to different histories of the particular samples collected (some were elutriated material; others were from WDFM cylinders or the bulk container; again others were from material collected in the elutriator which was returned to the bulk container for reuse since only limited amounts of NEFA and FA were available) and variations in particle sizes which affects the nickel content (Natusch and Wallace, 1974; Campbell et al., 1978; Fisher et al., 1978a). Realistic final values appear to be 6% Ni for NEFA and 0.3% Ni for FA. These values are supported by the data presented in Table 15. While the dose separation of the experimental design (high and low NEFA exposures; Ni in NEFA versus Ni in FA) was thus maintained, the degree of dose separation between nickel in NEFA and in FA apparently was lower for the chronic exposure than for the subchronic exposure.

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The reasons for this phenomenon are not known. Human error (inadvertent mixing of NEFA and FA) can be ruled out. This statement is based on the fact that the two NEFA exposure chambers for the high and low NEFA exposures and the labeled NEFA container were located in one room, and the FA exposure chamber with the labeled FA container in a different room. The paths of the two substances met periodically only briefly when the cylinders of the WDFMs were loaded, using a Carver press. Here, too, all containers, including the WDFM cylinders, were clearly labeled. Even if inadvertent mixing had occasionally occurred on such occasions, the resultant short (until the cylinder needed refilling) exposures to the wrong dust--brief compared to the total cumulative exposure time--could not account for the results in Table 14. Furthermore, a mix-up at the loading of the WDFM cylinders could not account for the changes in the bulk samples.

Assuming that the pulmonary clearance rate in the FA-exposed animals was the same as in the high NEFA-exposed animals, the data in the Ni/Lung column of Table 14 (725 μg for the high NEFA group vs. 36 μg for the FA group, adjusted for background levels of 6 μg in the controls) indicate for the chronic exposures a 20-fold higher nickel content in the NEFA than in the FA. These data are in reasonable agreement with the reanalysis data for leftover quantities of NEFA and FA, referred to above.

In summary, it is known that the mean concentrations of a number of elements in fly ash are inversely proportional to the particle size (Natusch and Wallace, 1974; Campbell et al., 1978; Fisher et al., 1978a), at least for certain size ranges. It has further been shown (Hayes et al., 1978) that even within a relatively narrow particle size range the concentration of a given element can vary greatly among individual particles; that the quantity of a given element, determined as the mean concentration by multiple-particle (bulk) analytical procedures, is normally concentrated at very high levels in only a very few particles, the remaining particles having very low or no detectable concentrations of the element; and, therefore, that estimates of toxicity based on bulk analysis data rather than on single particle analyses can be misleading. Finally, Fisher et al. (1978b) have undertaken physical and morphological studies of size-classified coal fly ash, in which they have identified eleven major morphological classes of coal fly ash.

This shows that the term "fly ash"--even when referring to one source material (either coal or oil) of the same origin (e.g., the same type of coal mined from the same seam)--refers to rather complex particles featuring many largely uncontrolled variables, including widely varying and different physical and chemical properties. It might thus be difficult to attribute a given toxic/pathogenic effect that one might observe as the result of exposure to fly ash, to any of its many and varying characteristics. Fortuitously, we were not placed in this position since our results were essentially restricted to an expected anthracosis, interstitial reaction and bronchiolization as a function of dust exposure level (i.e. dust quantity rather than its nickel content) with no differentiation in lesions between NEFA- and FA-exposed animals exposed to the same aerosol concentrations (~70 μg/ℓ).

Of importance beyond the scope of this study is to determine whether the phenomena discovered for fly ash (inverse proportionality of elemental concentration to particle diameter; great chemical heterogeneity among individual particles, with elemental concentrations in individual particles being dramatically different from the mean concentrations determined by bulk analysis) apply also to other particulate air pollutants because of the biological significance of these phenomena.

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ANCILLARY STUDIES

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I. PULMONARY DEPOSITION, TRANSLOCATION AND CLEARANCE OF INHALED NEUTRON-ACTIVATED FLY ASH IN HAMSTERS

ABSTRACT

To determine pulmonary deposition, translocation and clearance of inhaled fly ash, hamsters received a single 95-minute nose-only exposure to neutron-activated fly ash. The mean respirable aerosol concentration was 470 μg/ℓ. Over a period of 99 days postexposure, the hamsters were sacrificed in groups of six animals. Lungs, liver, kidneys, decapitated and skinned carcass, pelt, head, gastrointestinal tract, urine, and feces were collected for analysis of the radionuclide tracers ^{46}Sc, ^{59}Fe, and ^{60}Co by γ-ray spectrometry. The fly ash burden estimates as determined by the radionuclides ^{46}Sc and ^{59}Fe are in good agreement for the majority of samples analyzed. Such close agreement indicates fly ash particulate levels in the lungs, carcass, head, pelt, GI tract, and feces rather than leached radionuclides. Relative to the ^{46}Sc and ^{59}Fe-based estimates, fly ash deposition estimates obtained with the isotope ^{60}Co were appreciably lower for the lungs and appreciably higher for one or more sacrifice times for carcass, liver, head, pelt, and urine samples. This indicates that ^{60}Co (and thus the element cobalt) selectively leached from fly ash deposited in the deep lung, translocated to other sites, and was excreted in the urine.

An estimated average of 63 μg fly ash, or 2 to 3% of the inhaled fly ash, was initially retained in the respiratory tract. The estimated biological half-times of the fly ash were 2.6 and 34.5 days, probably for the airways and for the deep lung, respectively. After 99 days, the mean lung burden had decreased to about 10% of its initial value.

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INTRODUCTION

Inhalation exposures of laboratory animals to environmental or industrial air pollutants simulate more closely than other routes of administration the mode of human exposure to these agents. Inhalation exposures are, therefore, frequently used to determine the biological effects of an air pollutant--or a combination of air pollutants--in an animal model in an attempt to assess the risk of such exposures to man. Generally, such inhalation studies involve the exposure of animals to different dose levels of the agent(s) to be investigated, with body weight, life span and histologic findings serving as criteria to evaluate biological effects; sometimes the criteria are extended to include biochemical, physiological, or other tests.

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Regardless of the details of the experimental protocol for such studies, it is important to obtain information on the fate of the material deposited in the lungs of the exposed animals to correctly interpret results of the exposures. More specifically, it is of interest to determine that fraction of the inhaled material which is deposited in the deep lung, the biological half-life of the material in the lung, whether the material translocates from the lung to the other sites and, if so, the rate of translocation.

Pulmonary deposition, translocation and clearance studies, however, are often problematic in that only minute quantities of material are deposited in the deep lung, which decrease as a function of time as the material is cleared from the lung. Usually the elements to be analyzed also occur naturally at varying levels in animal tissues, further confounding analysis. The extremely sensitive technique of neutron activating the material before the animal exposure and subsequent γ-ray analysis of the tissue and excreta samples circumvents this analytical problem as the radioactively labeled material can be readily measured against a very low background in samples from control animals.

The objective of this study was to provide supplementary and complementary information to inhalation experiments with nickel-enriched fly ash by investigating pulmonary deposition, translocation and clearance characteristics of neutron-activated fly ash in our animal model, the hamster. Using neutron-activated fly ash for the inhalation exposure and γ-ray analysis of the tissues of interest, the fate of the material deposited in the animals was monitored for 99 days.

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CONCLUSIONS

The fly ash burden estimates as determined by the radionuclides ^{46}Sc and ^{59}Fe are in good agreement for the majority of samples analyzed. Such close agreement indicates fly ash particulate levels in the lungs, carcass, heald, pelt, GI tract, and feces rather than leached radionuclides. Relative to the ^{46}Sc and ^{59}Fe-based estimates, fly ash deposition estimates obtained with the isotope ^{60}Co were appreciably lower for the lungs and appreciably higher for one or more sacrifice times for carcass, liver, head, pelt, and urine samples. This indicates that ^{60}Co (and thus the element cobalt) selectively leached from fly ash deposited in the deep lung, translocated to other sites, and was excreted in the urine. Most of the urinary ^{60}Co excretion took place within the first few days after exposure.

An estimated average of 63 μg fly ash, or 2 to 3% of the inhaled fly ash, was initially retained in the respiratory tract. The estimated biological half-times of the fly ash were 2.6 and 34.5 days, probably for the airways and for the deep lung, respectively. After 99 days, the mean lung burden had decreased to about 10% of its initial value. Extrapolating from the clearance data, it can be estimated that near-complete clearance of fly ash from the lung would have been achieved approximately 200 days post-exposure.

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MATERIALS AND METHODS

NEUTRON ACTIVATION OF COAL FLY ASH

To provide the radioactive feedstock from which the experimental aerosol was to be generated, approximately 50 grams of coal fly ash* were prepared for neutron activation in Hanford's N-reactor. The feedstock fly ash was dried at 105°C to remove moisture and irradiated in five 10-gram aliquots in the core of the reactor. Each aliquot was encapsulated in a sealed quartz container and irradiated for two hours in a neutron flux of approximately 10^{14}n/cm^2/sec. Radionuclides were induced by (n,γ) reactions on the major and minor elements in the fly ash. Following the irradiations, the neutron-activated fly ash was stored for a period of about five weeks to allow complete or partial decay of the short-lived activation products.

At the end of the decay period, the five separate fly ash aliquots were recombined and the entire inventory was sieved through an 80-mesh screen to provide a uniform particle size distribution for subsequent homogenization. A gentle hand-grinding of a few larger aggregates was required to sieve all the irradiated material. To obtain a uniform specific γ-ray activity, the fly ash was then homogenized by mixing for several hours with a wrist-arm shaker device. Replicate 0.1-gram aliquots of the sieved and homogenized fly ash were then sampled and counted on a solid-state Ge(Li) detector to verify a homogenous specific γ-ray activity and to identify potential isotopes which could be used as tracers. Because of their intensities, relatively long half-lives and other favorable radioactive decay properties, the radionuclides ^{60}Co, ^{46}Sc, and ^{59}Fe were selected as the preferable fly ash tracers. The elemental concentrations of cobalt, scandium and iron in the feedstock fly ash were determined by instrumental neutron activation analysis and were found to be 39 ppm, 18.5 ppm, and 20% by weight, respectively. Following these preparations and evaluations, the irradiated fly ash was aerosolized for the animal exposure.

AEROSOL EXPOSURE SYSTEM

Exposure of the hamsters to the neutron-activated fly ash took place in our radioactive aerosol exposure laboratory (Figure A1). The 20-liter aerosol exposure chamber (Figure A2) was constructed of Lucite with exposure ports for nose-only exposures arranged in seven tiers, each containing 10

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* Prepared by Battelle Columbus Laboratories from Ohio coal, mined in Pittsburgh No. 8 seam, by methods described earlier in this report.

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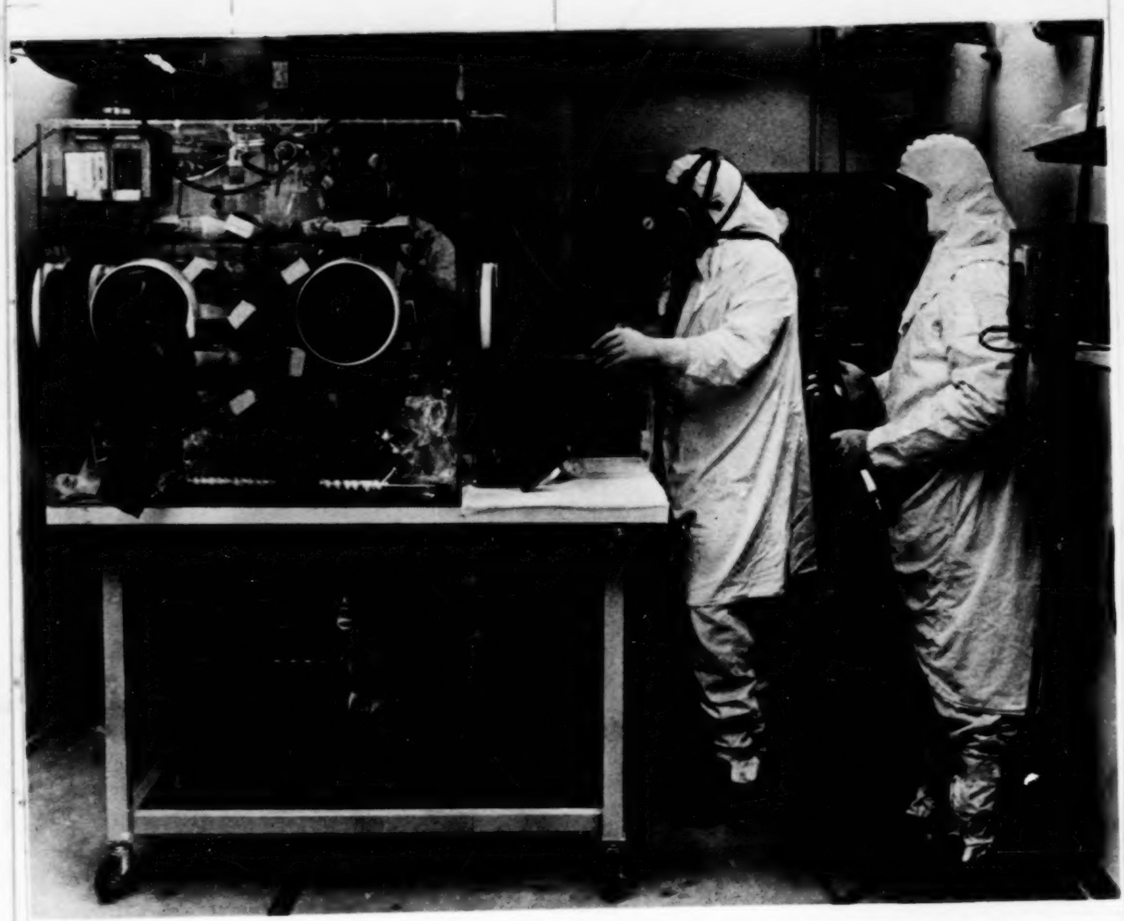

FIGURE A1. System for exposing rodents to radioactive aerosols.

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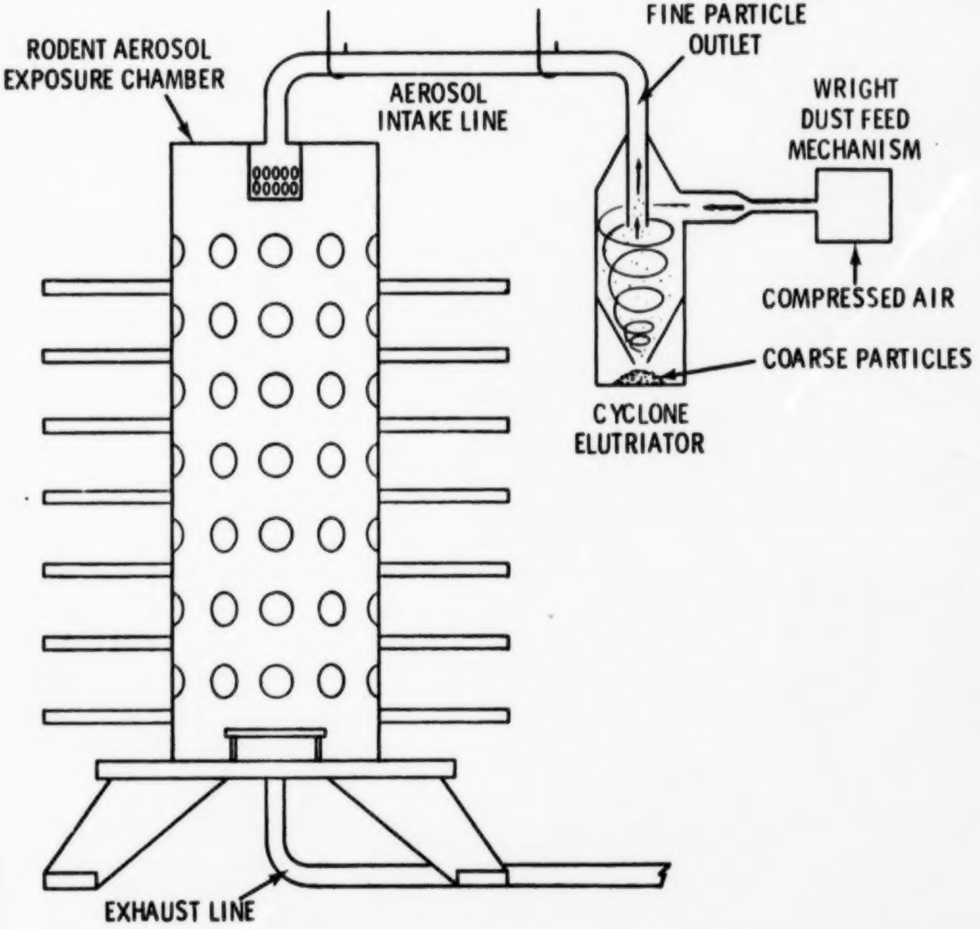

FIGURE A2. Schematic diagram of system for exposing rodents to radioactive aerosols.

exposure ports. For exposure, the animals were placed in soft-drink bottles that had the bottom and part of the top removed so that the noses of the animals were close to the open tops of the bottles. The animals were maintained in this position by wadding pushed against their posteriors, and by neoprene stoppers taped to the bottoms of, and sealing, the bottles. The bottles were inserted through neoprene stoppers into the ports of the exposure chamber. The bottled hamsters and four aerosol samplers were positioned on the lower six of the seven tiers. More specifically, three filter holders with Metricel DM-450 filters (0.45 μm mean pore size) were positioned on tiers 2, 4 and 6. The filters were weighed before and after sampling to estimate total aerosol concentration. An Andersen cascade impactor (Andersen, 1966) was positioned on tier 3 to allow estimations of the particle size distribution and the respirable aerosol concentration. The ports not occupied by animals or aerosol samplers were closed with neoprene stoppers.

The fly ash aerosol was generated by a Wright Dust Feed Mechanism (Wright, 1950) and then passed through a cyclone elutriator for removal of particles larger than approximately 10 μm aerodynamic equivalent diameter (AED). The aerosol exposure chamber, aerosol generating equipment and aerosol sampling devices were contained in a Lucite glove box under slightly negative pressure relative to the exposure room.

ANIMAL EXPOSURE, SERIAL SACRIFICES AND POSTMORTEM PROCEDURES

Sixty 5-month-old male Syrian Golden hamsters (*Mesocricetus auratus*, outbred LAK:LVG (SYR) Charles River Lakeview, Wilmington, MA), weighing from 120 to 130 grams, were prepared for nose-only exposure to the aerosol of neutron-activated fly ash as described. One hamster died in the restraint tube before exposure; another died during the 95-minute exposure, leaving a total of 58 animals to complete the exposure. The animals were of the same strain and were obtained from the same source as those in the inhalation studies with nickel-enriched fly ash described in the preceding part of this report. The aerosol data are summarized in Table A1. The mean respirable aerosol concentration was 470 μg/ℓ; the activity median aerodynamic diameter (AMAD) was approximately 3.5 μm, and the geometric standard deviation 2.8.

After exposure, the animals were serially sacrificed in groups of six by randomized selection of one animal from each of the six occupied tiers of the exposure chamber. Tissues and excreta were collected according to the schedule shown in Table A2. The lungs, including mediastinal tissue, liver, kidneys, decapitated and skinned carcass, pelt, head, gastrointestinal (GI) tract, urine, and feces were analyzed for radioactive isotopes by γ-ray spectrometry, and the γ-ray counts were compared with those obtained from 10 unexposed control hamsters. Five of the control animals were killed two days after the test animals were exposed; the remaining five control animals were killed 102 days after exposure of the test animals. The tissues from the control animals were analyzed like those from the exposed animals, together with urine and feces samples collected during the 2 days preceding the first sacrifice.

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TABLE A1. SUMMARY OF NEUTRON-ACTIVATED FLY ASH AEROSOL DATA

	60 Min	Total Counts	^{233}Pa 0.312MEV	^{51}Cr 0.320MEV	^{58}Co 0.811MEV	^{46}Sc 0.899MEV	^{59}Fe 1.099MEV	^{46}Sc 1.121MEV	^{60}Co 1.173MEV
Mean Aerosol Conc.* (μg/ℓ)	470								
S_x	39								
$S_{\bar{x}}$	23								
AMAD** (μm)		3.625	3.533	2.811	3.680	3.538	3.999	3.538	3.382
GSD		2.79	2.75	2.84	2.82	2.77	2.78	2.71	2.77

	^{59}Fe 1.292MEV	^{60}Co 1.332MEV	^{152}Eu 1.408MEV
AMAD (μm)	3.991	3.397	3.328
GSD	2.79	2.77	2.70

---

* Determined by means of filter paper samples at tiers 2, 4 and 6

** The activity median aerodynamic diameter (AMAD) was determined by means of a cascade impactor

S_x = Standard deviation

$S_{\bar{x}}$ = Standard error of the mean

GSD = Geometric standard deviation

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TABLE A2. SACRIFICE TIMES AND LOCATION OF ANIMALS IN EXPOSURE CHAMBER

EXPOSED ANIMALS

Time of Sacrifice after Exposure	Location of Animal During Exposure†					
	Tier 2	Tier 3	Tier 4	Tier 5	Tier 6	Tier 7
15 min	5	16	23	32	47	59
1 day	2	17	21	36	50	53
3 days	10	20	25	35	41	54
7 days	1	15	27	38	45	51
14 days	7	14	24	31	49	55
24 days	3	13	26	40	42	56
51 days	8	11	29	39	43	57
99 days††	4	18	28	33	44	52
9	12	22	34	48	60	
		30	37	46	58	

Total number of exposed animals = 58

CONTROLS

Time of Sacrifice after Exposure of Experimental Animals	Animal Number				
2 days	1	2	3	4	5
102 days	6	7	8	9	10

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† Numbers in tier columns are individual animal identification numbers

†† Of the surviving 16 animals sacrificed 99 days after exposure, six were randomly selected for γ-ray analysis

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Analyses were carried out on the lungs to determine pulmonary deposition and clearance, and on liver and kidneys to determine whether during translocation (if any) and clearance of pulmonary deposits of fly ash, the material would pass through and/or accumulate in these organs. The remainder of the skinned carcass was analyzed for fly ash with no attempt at further differentiation of translocation sites. The skin was removed from the carcass to reduce the chance of sample contamination. The head was analyzed separately to prevent the expected relatively heavy deposition on and in the head area from confounding the other deposition data. Analyzing excreta as part of a translocation and clearance study sheds light on the excretion rate of the material and aids in estimating initial deposition of the inhaled material.

ANALYSIS OF SAMPLES

Tissue samples for γ-ray counting were preserved by freezing. The small tissue samples (lung, kidneys, and liver) and fecal samples were transferred directly into vials for counting. Large samples (carcass, head, GI tract, and pelt) required high-temperature ashing to reduce the volume for analysis. Previous work in our laboratory has shown that ashing does not cause losses of the radionuclides through volatilization (L. A. Rancitelli, unpublished data, 1975). The urine samples were transferred into polyethylene vials and evaporated to dryness with a heat lamp before analysis. The prepared samples were centered in 2-dram polyethylene vials for γ-ray counting.

The γ-ray analyses were performed on a Ge(Li) detector and several Na(Tl) γ-γ coincidence spectrometers. Feedstock fly ash standards in geometrical arrangements identical to those of the samples were counted on each detector system used for sample analysis. Counting times ranged from 30 to 2000 minutes, depending on the activity in the samples. The amount of fly ash in the samples was determined by comparing the samples with the feedstock standards.

The isotope ^{60}Co was used in all cases to calculate the fly ash in samples since it has a long half-life (5.2 years as compared to 83.8 days for ^{46}Sc and 45 days for ^{59}Fe) and easily detectable coincident γ-rays. For the period of this study, the decay correction for the ^{60}Co activity was 5% or less. In addition, ^{46}Sc and ^{59}Fe activities were used to estimate the quantity of fly ash for purposes of comparing the ^{46}Sc-based and ^{59}Fe-based values with the ^{60}Co-based values. The advantage of using more than one radioactive tracer to monitor translocation of fly ash lies in observing two or more elements with different dissolution and transfer coefficients in biological systems. If, for example, the ^{60}Co:^{46}Sc activity ratio in a biological tissue is identical to the ratio in the radioactive fly ash, it strongly suggests that the radioisotopes were transferred together by particulates. An alternative situation that would produce the same ^{60}Co:^{46}Sc ratio in tissue as was observed in the fly ash would require that these radionuclides be leached and transferred in identical fashion. This latter possibility is not supported by past experience in our laboratory, which

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suggests that biological systems take up Co in preference to Sc (L. A. Rancitelli, unpublished data, 1975).

RELATIVE VARIATIONS OF TRACER RADIONUCLIDE SPECIFIC ACTIVITIES AS RELATED TO PARTICLE SIZE

The elemental composition of coal fly ash is known to vary considerably as a function of particle size. For example, the abundance of several volatile trace metals has been shown to be highly enriched in the smaller particle size ranges (Campbell et al., 1978). Because of this, the specific activity of a given radionuclide tracer in the irradiated feedstock may be appreciably different from the specific activity of the same radionuclide in the experimentally generated aerosol or fractions thereof. These differences may result from: (1) changes in particle size distributions caused by the aerosol generating system (e.g., by the elutriator); and/or (2) particle size separations in the respiratory system of the experimental animals.

In this study, the measurement of deposited fly ash particulates was to be quantified by comparison to the known specific activities of the sieved and homogenized feedstock. However, if during aerosol generation or inhalation the particle size distribution changed, the original elemental abundances of cobalt, iron and scandium in the prepared feedstock fly ash might also be changed and thus compromise the normalization to the "standard" specific activities of the feedstock samples. Therefore, the possible differences in specific activities for the feedstock and derivative aerosol fractions needed to be evaluated to preserve the accuracy of the intended quantitative analysis.

To resolve this potential problem, samples of the experimentally derived fly ash aerosol were collected on preweighed 0.45-μm Millipore filters and on preweighed stages of an Andersen cascade impactor. The aerosolized fly ash material that was collected was weighed and then counted on the same Ge(Li) detector that had been used previously for the 0.1-gram feedstock standards. The resulting data, including those of the feedstock standards, are tabulated in Table A3.

To evaluate the specific counting activity of each radionuclide in the experimental aerosol relative to its activity in the original feedstock fly ash, ratios were computed using the mean values in Table A3. The measure of difference was calculated as the ratio of the sum of means for the two energies associated with a given isotope for the aerosol (or indicated fraction) to the sum of means for the feedstock standard. The calculated ratios are given in Table A4. Estimates of the standard deviation of these ratios were approximated by propagation of error techniques, and approximate 95% confidence bounds were calculated for each ratio. The ratios for which the confidence interval did not include 1.0 (equality of activity in aerosol and feedstock) are indicated.

These results imply that: (1) the respirable fraction of the aerosol (<4.8 μm) had a significantly lower specific activity for the radionuclides

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TABLE A3. SPECIFIC COUNTING ACTIVITIES (± 1 σ COUNTING ERRORS)* OF FEEDSTOCK AND AEROSOL FLY ASH SAMPLES (CPM/g)

		Isotope and Energy (MEV)					
		^{60}Co	^{60}Co	^{59}Fe	^{59}Fe	^{46}Sc	^{46}Sc
Material		1.173	1.132	1.099	1.292	0.889	1.121
Feedstock Fly Ash Standard #1		3640 ± 69	3298 ± 61	26,491 ± 180	17,582 ± 142	26,510 ± 201	21,475 ± 170
Feedstock Fly Ash Standard #2		3764 ± 88	3272 ± 78	26,748 ± 235	17,640 ± 185	27,173 ± 258	21,587 ± 212
Feedstock Fly Ash Standard #3		3740 ± 80	3300 ± 71	26,779 ± 230	17,852 ± 166	26,871 ± 223	21,981 ± 191
Mean ± SD		3715 ± 65.8	3290 ± 15.6	26,673 ± 158	17,691 ± 142	26,851 ± 332	21,681 ± 266
Fly Ash Aerosol #1 (tier 2)		3835 ± 110	3510 ± 98	24,170 ± 266	16,198 ± 211	26,976 ± 310	21,249 ± 263
Fly Ash Aerosol #2 (tier 4)		3886 ± 113	3424 ± 102	23,976 ± 269	15,639 ± 214	25,994 ± 315	21,005 ± 265
Fly Ash Aerosol #3 (tier 6)		4183 ± 119	3444 ± 103	24,162 ± 280	15,993 ± 222	26,667 ± 328	21,073 ± 276
Mean ± SD		3968 ± 188	3459 ± 188	24,103 ± 110	15,943 ± 283	26,546 ± 502	21,109 ± 126
Fractionated Fly Ash Aerosol							
Impactor Stage	Size Range (μm)						
#4	3.12 - 4.80	3541 ± 73	3190 ± 65	23,321 ± 182	15,323 ± 144	24,424 ± 208	19,547 ± 174
#5	2.41 - 3.12	3487 ± 63	3068 ± 57	18,923 ± 148	12,527 ± 115	22,283 ± 176	18,141 ± 151
#6	0.99 - 2.41	3393 ± 78	2984 ± 69	17,100 ± 174	11,286 ± 138	21,788 ± 216	17,365 ± 186
#7	0.64 - 0.99	3423 ± 84	2967 ± 75	16,353 ± 185	10,990 ± 146	21,702 ± 232	17,333 ± 196
#8	0.43 - 0.64	3923 ± 56	3475 ± 51	17,156 ± 118	11,324 ± 93	22,626 ± 147	18,037 ± 126
#9	<0.43	3546 ± 82	3131 ± 74	15,086 ± 169	10,418 ± 136	21,512 ± 219	17,533 ± 189
Mean #4 through #9 ± SD		3552 ± 192	3136 ± 187	17,990 ± 2895	11,978 ± 1778	22,389 ± 1078	17,993 ± 834

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*Calculated as $\frac{\sqrt{\text{Gross Count}}}{\text{Net Count}}$; gross count = total count, net count = gross count minus background count

TABLE A4. RATIO (± SD) OF TRACER RADIONUCLIDE COUNTING ACTIVITIES IN FLY ASH AEROSOL TO FEEDSTOCK FLY ASH

Ratio	^{46}Sc	^{59}Fe	^{60}Co
$\frac{\text{Sub-4.8μm Fraction}}{\text{Feedstock}}$	0.832* ± 0.0400	0.675* ± 0.105	0.955 ± 0.0543
$\frac{\text{Total Aerosol}}{\text{Feedstock}}$	0.982 ± 0.0244	0.903* ± 0.0104	1.060* ± 0.0263

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* An approximate 95% confidence interval does not include the expected ratio of 1.0.

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^{46}Sc and ^{59}Fe; (2) the total aerosol had significantly higher and lower specific activities for ^{60}Co and ^{59}Fe, respectively. These variations are due to the anticipated changes in elemental concentrations as a function of particle size. To correct for their source of error in each radionuclide tracer, the ratios in Table A4 were applied as corrective factors to ensure an accurate quantitative analysis of deposited fly ash particles as measured by each isotope. The ratios representing the respirable fraction were applied to the lung tissue samples. The ratios associated with the unfractionated or "whole" fly ash aerosol were applied to all other animal tissue and excreta samples.

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RESULTS AND DISCUSSION

The fly ash burdens as a function of time in the tissues and excreta of the exposed hamsters and the background values for the control animals are tabulated in Table A5. Examination of the ^{46}Sc-, the ^{59}Fe-, and the ^{60}Co-based lung values shows good agreement between the ^{46}Sc-and the ^{59}Fe-based data. This indicates that these values reflect fly ash particles rather than leached radionuclides. The ^{60}Co-based lung values, on the other hand, are substantially lower than those based on the other two radionuclides. This suggests that ^{60}Co leached selectively from the fly ash deposited in the deep lung, translocating primarily to the head and the pelt, as indicated by the consistently and markedly higher ^{60}Co values for these samples. Previous work in our laboratory with similar findings (Wehner et al., 1977a, b; Wilkerson et al., 1977), significantly higher ^{60}Co values for the urine samples, and the increased ^{60}Co values for the head and pelt support the statement that leaching of ^{60}Co occurred. Apparently, leaching and translocation of ^{60}Co from the lung occurred rapidly as indicated by the high ^{60}Co values for the first (15-minute postexposure) sacrifice samples for kidneys, liver and carcass. The leached ^{60}Co was excreted in the urine, and most of the urinary ^{60}Co excretion took place within the first few days after exposure.

Figure A3 shows the pulmonary deposition and clearance of the fly ash, based on the data from ^{46}Sc. The estimated curve is within one standard error of the mean (SEM) for all sacrifice times except 7 days (at 7 days the predicted value is within 2 SEM), indicating a good fit to the data. A nonlinear algorithm was used to fit a two-compartment model to the ^{46}Sc and the ^{59}Fe-based lung burdens in Table A5 as a function of time. The fits were preferable over their single compartment counterparts by minimizing the residual sum of squares. Similarly, for ^{46}Sc, the fit was slightly preferable to the ^{59}Fe modeling. The function for ^{46}Sc was

$$Y = 26.5\ e^{-0.268t} + 36.7\ e^{-0.0201t}. \qquad (1)$$

This model estimates the biological half-times to be 2.6 ± 3.2 and 34.5 ± 28.7 days (mean ± standard deviation), the sizable standard deviations reflecting the variability in the data. The shorter biological half-time of 2.6 days probably reflects material cleared relatively quickly by mucociliary action, whereas the half-time of 34.5 days reflects the deep lung burden (alveolar deposition). At 99 days postexposure, the mean lung burden had decreased to about 10% of its initial value. Extrapolating from (1), it can be estimated that near-complete clearance and/or translocation of fly

TABLE A5. FLY BURDENS IN TISSUES AND EXCRETA AS A FUNCTION OF TIME AFTER EXPOSURE (ALL VALUES ARE IN μg AND REPRESENT THE MEAN ± ONE SD* OF SIX HAMSTER SAMPLES.)

Time of Sacrifice after Exposure	Lungs			Head		
	^{46}Sc	^{59}Fe	^{60}Co	^{46}Sc	^{59}Fe	^{60}Co
15 minutes	65.71 ± 49.30	63.19 ± 48.88	40.1 ± 33.1	101.7 ± 57.2	103.2 ± 50.4	120.2 ± 55.3
1 day	48.82 ± 15.24	48.44 ± 13.64	13.0 ± 7.0	20.3 ± 10.8	21.8 ± 10.6	46.5 ± 25.7
3 days	55.67 ± 28.30	55.40 ± 25.99	25.6 ± 13.6	6.1 ± 2.6	9.2 ± 3.4	25.7 ± 19.3
7 days	27.91 ± 10.64	28.98 ± 11.64	11.5 ± 5.5	2.0 ± 1.1	4.7 ± 2.7	22.0 ± 12.8
14 days	34.07 ± 16.49	37.30 ± 16.69	14.1 ± 7.2			
24 days	20.68 ± 9.25	24.21 ± 10.33	8.3 ± 4.4			
51 days	12.15 ± 10.30	14.48 ± 11.46	5.2 ± 5.0			
99 days	6.38 ± 4.44	<7.65 ± 5.52	2.4 ± 1.7			
Controls						
2 days	<0.07 ± 0.03	<1.2 ± 0.13	<0.21 ± 0.09	<0.08 ± 0.01	<0.81 ± 0.20	<0.26 ± 0.07
102 days	<0.09 ± 0.03	<3.6 ± 1.6	<0.14 ± 0.08			

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* The standard deviations associated with means reported as "less than" were estimated on limited (<) data and are, therefore, only approximate. If any individual value(s) upon which the mean is based is a limited (<) number, the mean is reported as a limited (<) number.

The differences in the means reported as "less than" (ranging from < 11.3 [GI tract; ^{60}Co 7-day postexposure value] to 0.05 [^{46}Sc value for urine of controls]) are mainly due to different counting times, resulting in different sensitivities.

TABLE A5. (CONTINUED)

Time of Sacrifice after Exposure	Liver			Kidneys		
	^{46}Sc	^{59}Fe	^{60}Co	^{46}Sc	^{59}Fe	^{60}Co
15 minutes	0.52 ± 0.45	1.2 ± 1.0	3.4 ± 1.9	<0.12 ± 0.07	<0.40 ± 0.06	<0.94 ± 0.48
1 day	0.41 ± 0 .19	2.7 ± 1.9	1.9 ± 0.59	0.18 ± 0.09	<0.51 ± 0.05	0.49 ± 0.21
3 days	0.62 ± 0.27	3.1 ± 1.1	1.6 ± 0.54	<0.25 ± 0.12	0.43 ± 0.12	0.43 ± 0.12
7 days	0.35 ± 0.17	2.5 ± 1.2	1.0 ± 0.50	0.13 ± 0.09	<0.65 ± 0.08	0.38 ± 0.14
14 days	0.53 ± 0.26	4.1 ± 1.8	0.86 ± 0.27	<0.19 ± 0.09	<0.85 ± 0.43	<0.28 ± 0.10
24 days	0.48 ± 0.24	2.9 ± 1.2	0.79 ± 0.34	<0.08 ± 0.03	<1.2 ± 0.45	<0.18 ± 0.04
51 days	0.59 ± 0.50	<2.2 ± 0.99	0.48 ± 0.42	<0.09 ± 0.03	<1.3 ± 0.15	<0.20 ± 0.04
99 days	0.36 ± 0.24	<2.6 ± 1.2	<0.21 ± 0.09	<0.21 ± 0.31	<2.3 ± 0.23	<0.17 ± 0.05
Controls						
2 days	<0.04 ± 0.004	<0.75 ± 0.22	<0.16 ± 0.04	<0.04 ± 0.02	<0.78 ± 0.08	<0.16 ± 0.05
102 days	<0.05 ± 0.02	<3.8 ± 1.3	<0.13 ± 0.02	<0.07 ± 0.02	<2.6 ± 1.2	<0.15 ± 0.03

TABLE A5. (CONTINUED)

Time of Sacrifice after Exposure	Carcass ^{46}Sc	Carcass ^{59}Fe	Carcass ^{60}Co	Pelt ^{46}Sc	Pelt ^{59}Fe	Pelt ^{60}Co
15 minutes	26.9 ± 13.0	28.0 ± 14.6	66.9 ± 26.1	80.6 ± 39.1	75.8 ± 37.9	91.6 ± 43.6
1 day	10.0 ± 8.2	11.4 ± 7.9	14.9 ± 9.7	23.5 ± 12.6	21.5 ± 12.2	34.1 ± 17.2
3 days	6.0 ± 3.4	10.0 ± 3.6	8.8 ± 2.8	8.8 ± 2.3	9.2 ± 2.6	37.9 ± 30.0
7 days	1.7 ± 1.7	4.0 ± 1.3	2.4 ± 1.1	2.9 ± 2.0	4.4 ± 2.4	18.0 ± 14.3
14 days	1.5 ± 0.84	5.7 ± 2.3	2.2 ± 0.74			
24 days	<0.82 ± 0.49	<4.5 ± 1.5	1.3 ± 0.75			
51 days	<0.61 ± 0.54	<3.6 ± 2.0	<0.67 ± 0.54			
99 days	0.70 ± 0.37	<4.3 ± 1.3	0.54 ± 0.30			
Controls						
2 days	<0.08 ± 0.02	<0.86 ± 0.10	<0.27 ± 0.08	< 0.08 ± 0.02	<0.70 ± 0.05	<0.26 ± 0.10
102 days	<0.10 ± 0.02	<3.2 ± 1.2	<0.19 ± 0.04			

Table A5. (Continued)

Time of Sacrifice after Exposure	GI Tract		
	^{46}Sc	^{59}Fe	^{60}Co
15 minutes	813.5 ± 244.3	791.8 ± 232.9	767.2 ± 212.0
1 day	258.9 ± 107.9	228.0 ± 139.5	270.4 ± 105.6
3 days	57.6 ± 55.4	59.6 ± 57.3	67.6 ± 52.0
7 days	<4.3 ± 1.1	<5.3 ± 1.6	<11.3 ± 3.3
14 days	4.7 ± 0.71	<5.7 ± 2.4	<5.8 ± 2.1
24 days			
51 days			
99 days			
Controls			
2 days	<1.6 ± 0.41	<2.0 ± 0.93	<3.4 ± 1.2
102 days			

TABLE A5. (CONTINUED)

Time of Sacrifice after Exposure	Feces ^{46}Sc	Feces ^{59}Fe	Feces ^{60}Co	Urine ^{46}Sc	Urine ^{59}Fe	Urine ^{60}Co
15 minutes						
1 day	873.3 ± 339.6	831.1 ± 327.3	903.3 ± 126.9	1.0 ± 0.7	3.2 ± 0.33	21.3 ± 10.6
3 days	482.5 ± 358.0	471.1 ± 348.9	463.6 ± 306.0	0.88 ± 0.74	<3.2	16.8 ± 14.2
7 days	127.0 ± 168.4	130.3 ± 173.3	142.2 ± 179.5	0.9 ± 1.6	<3.5 ± 0.18	3.8 ± 4.2
14 days						
24 days						
51 days						
99 days						
Controls	<2.4 ± 1.3	<4.2 ± 1.5	<6.8 ± 4.3	<0.05 ± 0.02	<4.2 ± 0.22	< 0.13 ± 0.01

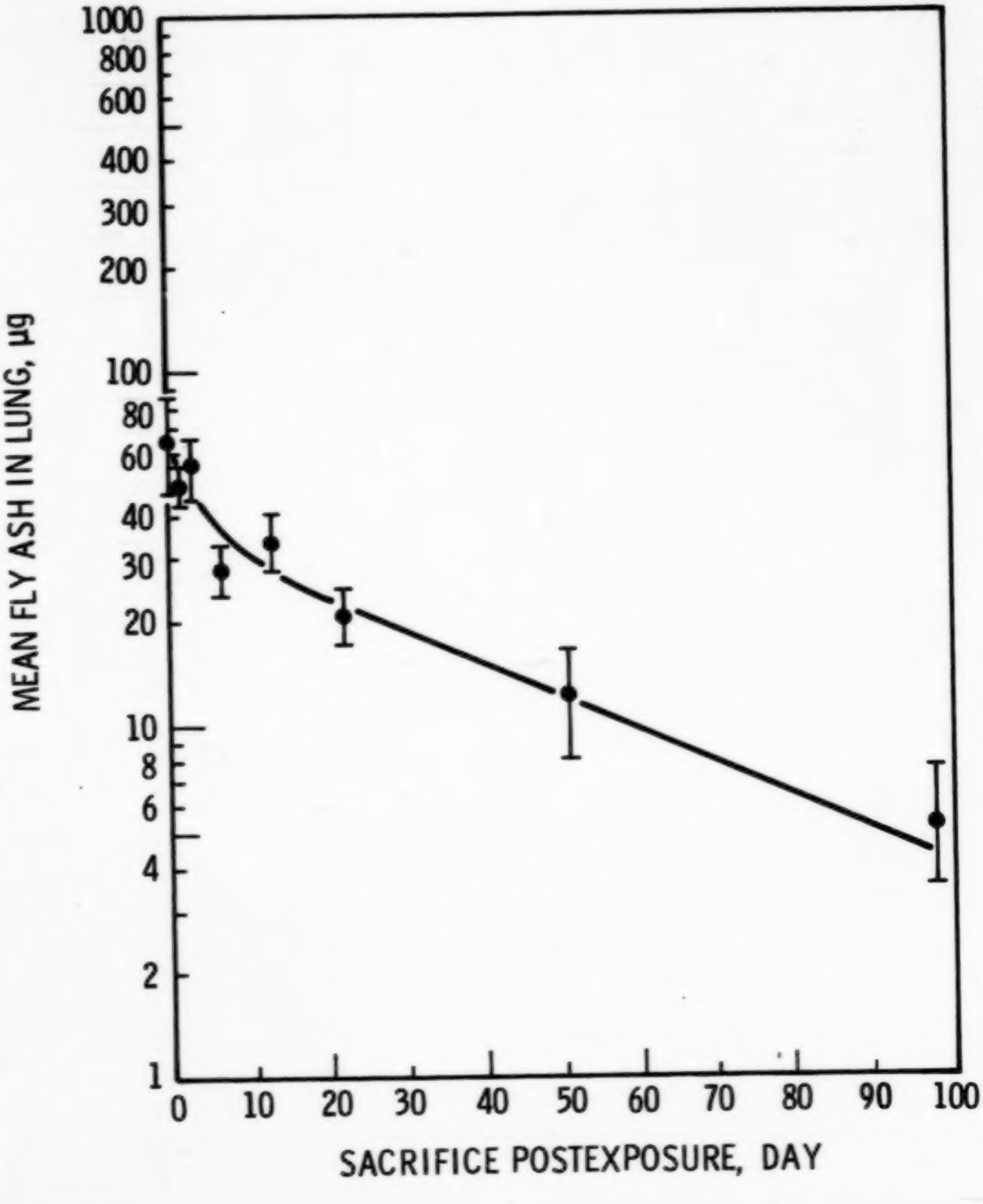

FIGURE A3. Pulmonary clearance of inhaled fly ash in hamsters (mean ± SEM) based on six animals per sacrifice time.

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ash from the lung would have been achieved approximately 200 days postexposure, at which time only about 1% of the lung burden is predicted to be retained in the lung. From (1), an estimate of the initial lung burden is 63.2 ± 8.3 µg.

The percentage of inhaled fly ash that was deposited and retained in the respiratory tract may be estimated from the following known parameters: respirable aerosol concentration (470 µg/ℓ); exposure time (95 minutes); minute volume of the hamster (60 ml/min*[Gleysteen and Stroud, 1974]); and the quantity of fly ash deposited in the respiratory tract of the exposed animals (~63 µg). These calculations indicate that approximately 2.7 mg of fly ash were inhaled during the 95-minute exposure. Of this quantity, about 63 µg, or 2.3%, were retained. This estimate is in good agreement with estimates of slightly less than 2% retention of inhaled nickel-enriched fly ash during the 6-hour acute toxicity test at a respirable aerosol concentration of ~220 µg/ℓ, and of between 3 and 4% during the forty 6-hour exposures (respirable aerosol concentration ~190 µg/ℓ) of the subacute toxicity test (Wehner et al., 1979).

Results of the acute toxicity test showed no appreciable decline in lung burden between 7 days (~78 mg) and 30 days postexposure, which might suggest a rather long biological half-time of nickel-enriched fly ash retained in the hamster lungs (Wehner et al., 1979). However, the 7-day value of 78 µg was based on only two animals. The present data indicate biological half-times of 2.6 and 34.5 days for fly ash, probably deposited in the airways and in the deep lung, respectively. Available evidence suggests that there should be no difference in pulmonary deposition, retention and clearance characteristics of fly ash particles and nickel-enriched fly ash particles (Wehner et al., 1979). Given the larger data base for the results of this pulmonary clearance study, it is reasonable to accept as valid the half-time values of 2 to 3 days for the airways and about 35 days for the deep lung.

The head samples showed a deposition of more than 100 µg fly ash. This quantity combines external deposition on the head and on or about the nares, and internal deposition in the nasopharyngeal area, plus some material probably in the process of being cleared by mucociliary action from the lower airways. This appreciable quantity, comprising both externally and internally deposited fly ash in an indistinguishable manner, would have confounded the carcass and pelt deposition data. For this reason the head was analyzed separately. Within 7 days after exposure, the head sample values had decreased to less than 5% of their original values as indicated by the ^{46}Sc and ^{59}Fe data. The elevated ^{60}Co data, whose difference from the ^{46}Sc and ^{59}Fe data increased as a function of time, can again be attributed to leaching of ^{60}Co from the fly ash. The reasons for the preferred translocation of ^{60}Co to the head--and also to the pelt--are not known. Initially high deposition due to the head's exposed position during exposure, and subsequent rapid clearance due to natural clearance mechanisms and the animals' grooming themselves, was expected.

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*Not allowing for possible changes in breathing pattern due to confinement of the animals in the restraint tubes during the 95-minute exposure.

What has been said about the head samples holds largely true for the pelt samples, too.

The deposition in the liver samples from exposed animals was compared to control animals at both sacrifice times by a two-tailed Mann-Whitney U test. The significances are indicated in Table A6. It was assumed that the estimated contents could be ranked by the reported measurements, even though some values are reported as upper limit values. In the case of ties, a conservative approach was taken so that the probabilities are at least as small as indicated. The ^{46}Sc- and ^{60}Co-based fly ash estimates for the liver samples of exposed hamsters were generally higher than those for both groups of controls, which were similar to one another. In the case of ^{59}Fe, the control estimates at 102 days (an upper limit is reported) were considerably higher than at 2 days. For ^{59}Fe, the 3-day and later sacrifice estimates differed from the estimates for the 2-day sacrificed controls, and the 15-minute sacrifice estimates differed from the estimates for the 102-day sacrificed controls. These are not the most reasonable comparisons when considering contiguous times of sacrifice. Thus, it can be concluded that the ^{59}Fe tracer data do not necessarily support the hypothesis of translocation of fly ash from the lung to the liver, whereas both the ^{46}Sc and ^{60}Co data do.

The deposition in the kidneys was not treated statistically due to the large number of values which were reported only as an upper bound and because the amounts in the exposed animals were so similar to the values observed in the control animals.

In both the liver and kidneys, the ^{60}Co values were highest for the first sacrifice and decreased steadily thereafter until they approached control levels. This suggests passage of leached ^{60}Co through these organs and is further evidence for the selective leaching of ^{60}Co from fly ash particles deposited in the hamsters.

The most plausible explanation for the values from the skinned carcass (minus head, liver, kidneys and GI tract) being higher than the control values for the first few sacrifices is the external deposition of fly ash particles on feet and on or between toes from where they were not removed with the pelt. The mean for the 15-minute sacrifice ^{60}Co values is more than twice as high as the means of the ^{46}Sc and ^{59}Fe samples. This might reflect the rapid leaching of ^{60}Co from the fly ash in the lung and subsequent translocation to the carcass.

Considerable quantities of fly ash were found in the GI tracts of the first groups sacrificed. The means (^{46}Sc and ^{59}Fe data only) decreased from roughly 800 μg to 250, 60, and 5 μg for the 15-minute, 1-day, 3-day, and 7-day sacrifices, respectively. These values combine material initially deposited in the respiratory tract and subsequently translocated by mucociliary clearance mechanisms to the GI tract, and externally deposited fly ash which was translocated to the GI tract by grooming (licking and swallowing) of the animals after exposure.

TABLE A6. SIGNIFICANT DIFFERENCES BETWEEN FLY ASH ESTIMATED IN LIVER OF EXPOSED AND CONTROL ANIMALS BASED ON RADIONUCLIDE TRACERS

Time of Sacrifice After Exposure	^{46}Sc Control Sacrifice Time 2 Days	^{46}Sc Control Sacrifice Time 102 Days	^{59}Fe Control Sacrifice Time 2 Days	^{59}Fe Control Sacrifice Time 102 Days	^{60}Co Control Sacrifice Time 2 Days	^{60}Co Control Sacrifice Time 102 Days
15 Minutes	**	**		*	**	**
1 Day	**	**			**	**
3 Days	**	**	**		**	**
7 Days	**	**	**		**	**
14 Days	**	**	**		**	**
24 Days	*	*	**		**	**
51 Days	**	**	**			**
99 Days	**	**	**			**

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* $p \leq 0.05$ based on two-tailed Mann-Whitney U test.
** $p \leq 0.01$ based on two-tailed Mann-Whitney U test.

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The values for the fecal samples reflect the GI burdens, decreasing (^{46}Sc and ^{59}Fe data only) from about 850 μg to 480 and 130 μg for the day 1, day 3, and day 7-samples, respectively. As can be expected for fecal samples, the values varied considerably about the means as indicated by the large standard deviations. The importance of the urine data lies in the high ^{60}Co values, indicating selective leaching of ^{60}Co from the fly ash particles, as pointed out before.

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II. AMES ASSAYS OF NICKEL-ENRICHED AND REGULAR FLY ASH

II. AMES ASSAYS OF NICKEL-ENRICHED AND REGULAR FLY ASH*

Dimethylsulfoxide (DMSO) extracts of NEFA and FA were tested for mutagenicity in the postmitochondrial/*Salmonella* (AMES) assay. The tests were carried out both with and without metabolic activation, using Aroclor-induced hepatic enzyme preparations as the source of activating enzymes. Two strains, *Salmonella typhimurium* TA 98 (frameshift mutant) and TA 100 (point mutant) were exposed to the DMSO extracts of NEFA and FA. The total concentration of FA and NEFA in the DMSO solvent was about 50 mg per ml. The concentrations used in the Ames assays of NEFA and FA ranged from 10 to 5000 μg per petri plate (concentrations included suspended plus extracted, solubilized materials). In all cases, the responses were negative. No increase above background mutation rates were observed for either TA 98 or TA 100. Thus, NEFA and FA do not appear to be mutagenic in the *standard* Ames assay. However, this does not preclude the possibility of demonstrating mutagenic activity by altering the standard Ames test in some way or by use of different extraction procedures to remove potential mutagens from the surface of NEFA and FA.

The negative findings presented here do not necessarily contradict the positive Ames test results of Chrisp et al. (1978), Fisher et al. (1979) and Fisher and Chrisp (1978) obtained with similar fly ash materials. These authors extracted their fly ash particles with horse serum, phosphate saline or cyclohexane but not DMSO. The horse serum extracts were the most mutagenically active of their extracts. It is possible that modifications of the extraction procedures used for NEFA and FA to include similar extractions, would also yield positive Ames test data.

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* Courtesy of Dr. Richard A. Pelroy.

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